Food Research

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Flavors in Foods — A Cumulative Review

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Grapefruit juice	10.0	49.0
Orange juice	9.7	70.0
Pineapple juice	5.4	18.0
Apple juice	0.2	3.6
Grape juice	0.0	4.7
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ROLE OF AMINO ACIDS IN THE BROWNING OF ORANGE JUICE

M. A. JOSLYN

Department of Food Technology, University of California, Berkeley

(Manuscript received June 13, 1956)

In orange juice, the constituents believed to be involved in browning are the reducing sugars, carboxylic acids, galacturonic acid, ascorbic acid, and amino acids. Although it can be demonstrated that, in relatively high concentration and high temperatures, galacturonic acid and glucuronic acid form colored compounds at a rate exceeding that found with common sugars *(39)*, it is unlikely that this reaction would be significant in browning orange juice at moderate temperatures. Similarly the demonstration that organic acid such as citric acid at pH close to neutrality would react with glucose at 100° C. to produce carbon dioxide and color does not necessarily imply that this reaction would occur in orange juice at pH 3.7 at moderate temperatures *(26)*. It is known that ascorbic acid at moderate temperatures either alone or in presence of added amino acids will react under both aerobic and anaerobic conditions to produce brown pigments *(1-3, 5-6, 9, 11, 16-18, 20, 24-25, 35-36)*. It is not known, however, whether decomposition of ascorbic acid alone or in presence of the sugars and amino acids of orange juice is more important than the sugar-amino acid reaction in browning. Model systems analogous to orange juice are not readily prepared since the actual amino acid composition of orange juice is unknown. The total amino acid content *(21, 34)* and the content of several amino acids are known *(28, 34)* but the many amino acids present have been detected only by qualitative paper chromatography *(28, 37, 42, 43)*. Which of the amino acids present is involved in the browning reaction until recently was not known. In the older investigations *(21, 27, 34)*, such evidence as was available did not indicate a decrease in amino acid content during the initial browning whether determined by fractional precipitation of amino acids *(34)* before and after browning, by Van Slyke and formol titration *(21)*, or by paper chromatography (*42*). It is now known, however, that these methods may be in error because the primary addition products of amino acids and sugars are ninhydrin reacting *(7)* and so may be included in the usual paper chromatography procedures as amino acids, and may react with nitrous acid in the Van Slyke determination *(17)*.

Degradation of amino acids by oxidized ascorbic acid to aldehydes, carbon dioxide and ammonia was reported by Abderhalden *(1-3)* to occur at pH 7 at 37° C. and Schönberg *et al. (38)* showed that unsubstituted α-amino acids undergo the Strecker degradation on heating with dehydroascorbic acid with the evolution of CO_2 and formation of aldehydes. On the other hand, ascorbic acid is known to react with alloxan and ninhydrin at pH 7.2 and 30° C. to give dehydroascorbic acid which then loses CO_2 and is converted to L-xylosone *(33)*. In the Strecker degradation the yield of aldehyde reported was small, varying from 5 to 10% but in the latter reaction almost quantitative yields of CO_2 were reported. Apparently the

type of nitrogenous constituent present influences the course of the reaction.

Oxidation of ascorbic acid is known to be involved in the browning of orange juice but the extent of oxidation required for appreciable browning varies with storage conditions *(8-10, 27, 30)*. At storage temperatures of 50° C. and above, darkening occurs in the presence of relatively small quantities of oxygen after only a 10-15% decrease in ascorbic acid content. At room temperature the extent of oxidation of ascorbic acid is appreciably greater. The method of measuring intensity of browning also is involved. The relation between rate of decrease in iodine reducing value and increase in optical density at two wave lengths (480 and 600 mμ) taken from the original data of Hamburger *(15)* is shown in Figure 1. This clearly shows

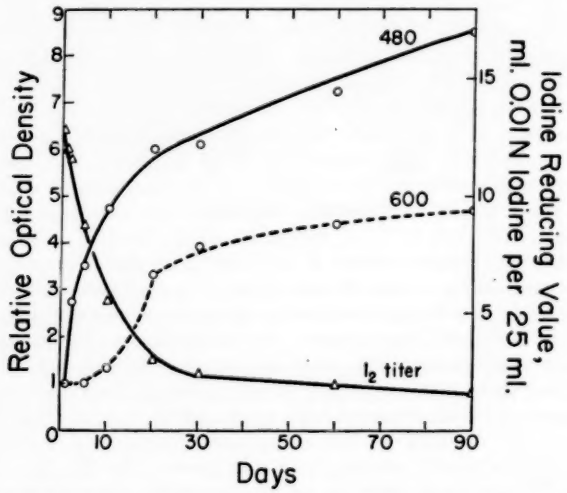

Figure 1. Relation between decrease in iodine reducing value (ml. of 0.01 N iodine solution per 25 ml.) and increase in optical density during oxidative browning of filtered orange juice at room temperature, 70° F. ± 2.

that after an initial lag the relative optical density increases rapidly during the period of rapid decrease in iodine reducing value but the dependence of change with time of optical density varies with the wave length region selected. Thus at 480 mμ the lag is shorter and the initial rate of increase is faster than at 600 mμ. This relation is shown in another way in Figure 2 where it is clear that appreciable increase in browning does not begin to occur until after the iodine reducing value has decreased to about half its initial value for measurements made at 600 mμ, but is appreciable when less than 10% decrease has occurred when measurements are made at 480 mμ.

Oxidation is also known to be involved in accumulation of furfural and hydroxymethyl furfural in orange juice *(32)*. This may be due to the greater tendency of dehydroascorbic acid to form furfural and other carbonyl compounds.

Since oxidative browning of orange juice is technically important, particularly in bottled packs, we have continued investigations of this and

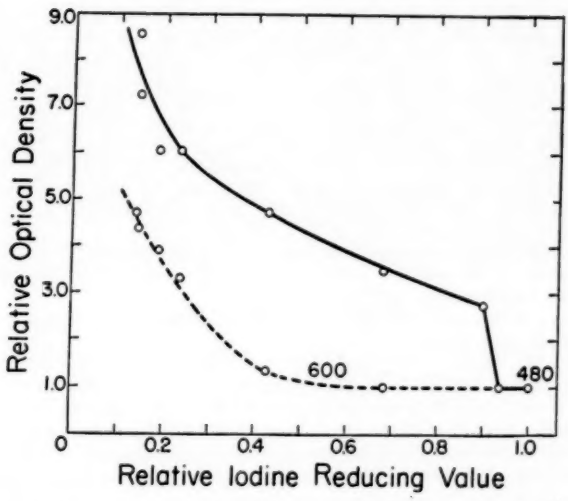

Figure 2. Relation of relative increase in optical density to relative decrease in iodine reducing value during oxidative browning of filtered orange juice at room temperature.

report observations on chromatographic changes in amino acid constituents of orange juice allowed to brown in presence of air in continuation of previously reported data *(42)*. Data on the rate of browning of ascorbic acid in presence of added sugars and amino acids also are presented.

EXPERIMENTAL METHODS

Orange juice samples. Valencia orange juice samples originally prepared on November 7, 1949 were examined after storage at room temperature (20-25° C.) for 4 years. The juice was stored in cotton stoppered bottles with 0.3% sodium benzoate. One lot was hand reamed and strained through cheese cloth; the other lot was centrifuged to remove all suspended particles. During storage the suspended particles of fruit tissue settled out and the supernatant liquid cleared; precipitation of colloidal material occurred also in the centrifuged sample. The precipitate was separated by filtration, washed and dried at 70° C. *in vacuo*. Its uronide content, expressed as anhydrogalacturonic acid, was 11.80% for the strained juice and 12.60% for the centrifuged juice, indicating a high proportion of non-uronide material. These juice samples had been examined chromatographically initially and after storage for four months for the presence of amino acids to detect changes occurring during initial browning *(42)*.

In addition to these samples, freshly extracted Washington Navel orange juice was prepared for fractionation studies. In our early investigations *(21)* the constituents present in orange juice were fractionally separated by precipitation with lead acetate at various pH values but the separations were not sufficient to identify the reactive constituents. Both the lead precipitable and lead soluble constituents after de-leading and adjusting to initial pH browned at about the same rate at room temperature.

Fractionation of reactive constituents of filtered orange juice by ion exchange resins, used in investigations of the browning of dried apricots *(4, 14)*, and carrots and potatoes *(44)* was used to obtain cationic, anionic and neutral fractions. Four lots of the juice, of initial pH 3.8, 11.1° Brix and titratable acidity of 0.983% as citric acid, were prepared: (1) untreated filtered orange juice with 0.2% sodium benzoate added

as preservative; (2) orange juice passed over Duolite CS100 cation exchange resin in the H-form to remove most of the nitrogenous constituents, the resin treated juice was adjusted to pH 3.8 with NaOH and sodium benzoate added; (3) orange juice passed over Duolite A-2 anion exchange resin in the OH form to remove acids including ascorbic acid and adjusted to pH 3.8 with HCl and 0.2% sodium benzoate; (4) orange juice passed over cation exchange resin and then over anion exchange resin to obtain a neutral fraction largely free of acidic and basic constituents. In this preliminary fractionation we did not elute the adsorbed fractions as Haas and Stadtman *(14)* did and did not analyze the fractions obtained. Twenty-five ml. aliquots of these samples were stored in partly filled cork stoppered test tubes (2.5 cm. × 20 cm.) at room temperature (20° C.) and in incubators at 30°, 40° and 60° C.

Ascorbic acid–amino acid–sugar solutions. To obtain additional information on the relative reactivity of the major constituents believed to be involved in browning, the following stock solutions were prepared:

Citric acid: C.P. citric acid hydrate 0.1 M, containing 0.2% sodium benzoate adjusted to pH 3.7 with KOH

Ascorbic acid: Merck U.S.P., 0.25 M in citrate buffer

Dextrose: Merck anhydrous reagent grade, 0.75 M in citrate buffer

Fructose: Pfanstiehl C.P. special grade, 0.75 M in citrate buffer

Amino acids: D-alanine (Nutritional Biochemicals), L-asparagine (Nutritional Biochemicals), L-glutamic acid (E.K. #1466), glycine (E.K. ammonia free #445), L-lysine monohydrochloride (Nutritional Biochemicals), 0.075 M in citrate buffer readjusted to pH 3.7.

Aliquots of these stock solutions, alone or in combination with citrate buffer were used to give reagent solutions containing 0.025 M ascorbic acid, 0.3 M sugar, and 0.03 M amino acid. Twenty-five ml. aliquots were dispensed into large cork stoppered test tubes (2.5 cm. O.D. × 20 cm.) having a headspace volume of 50 ml. and stored at 40° C.

To compare with these samples, the stock ascorbic acid solution was oxidized by treatment with activated carbon. Pure crystalline dehydroascorbic acid is still not readily available although its preparation by iodine oxidation *(23)* and bromine oxidation *(36)* has been described. Giriraj *(13)* reported that when 100 ml. of ascorbic acid solutions at pH 3.5 containing 50 mg. of ascorbic acid were treated with 10 g. of hydrochloric acid washed Norite carbon, above 95% of the ascorbic acid present was oxidized in 15 minutes and 90% of the original ascorbic acid could be recovered as dehydroascorbic acid. In our laboratories the dehydroascorbic acid obtained by the procedure of Kenyon and Munro *(23)* rarely assayed over 90% pure. It is known also that in solution, dehydroascorbic acid is rather labile and is transformed into 2:3-diketo-l-gulonic acid and other products *(13, 22)* and this conversion is influenced by sugars *(22)*. Therefore, for convenience in these preliminary investigations, 50 ml. of the stock ascorbic acid solution in citrate buffer were oxidized by mixing with 50 g. Darco G-60 carbon and after addition of 100 ml. of buffer the mixture was allowed to stand for 30 minutes and then washed with citrate buffer. The resulting filtrate was diluted to 500 ml. and then used in preparation of another lot of samples. In these the treated ascorbic acid solution also was adjusted to 0.025 M but the sugar concentration was 0.15 M and the amino acid concentration was 0.015 M. Fifty ml. aliquots of these solutions were stored in cork stoppered test tubes at 40° C. as above.

In addition solutions of 0.1 M citric acid buffer at pH 3.7 with ascorbic acid in the range of 0 to 0.063 M were prepared and 25 ml. aliquots stored at 40° C.

Chromatography. In the previously reported investigations orange juice has been applied either directly after filtration to filter paper *(42)* or after addition of sufficient alcohol to bring the alcohol content of the filtered extract to about 75% *(42)*. The sugars and other non-amino acid substances present resulted in appreciable "tailing" of some of the ninhydrin positive susbtances. Two dimensional paper chromatography was used with water saturated phenol in one direction and butanol-acetic acid-water (4:1:5) in the other.

Browning. In browning investigations several colorimetric and spectrophotometric methods have been used to measure the rate and extent of browning. These have included light reflectance and absorption measurements on the food products directly,

after filtration or extraction with water or solvent such as alcohol or acetone *(8, 9, 16, 17, 21, 27, 29-31, 35, 40)*. Curl *(9)*, for example, diluted 25-ml. aliquot of juice to 50 ml. with acetone and used the percentage transmission of the clear acetone solution as measured in an Evelyn photoelectric colorimeter with No. 420 filter as an index of color density; Lewis *et al. (26)* fractionated the pigments formed by acetone extraction and adsorption on alumina column and selected the fraction eluted with water as measure of browning. Although the pigments formed in browned orange juice have long been known to be quite complex and differ in solubility and other properties *(21)* for comparative purposes fractionation of these is not necessary. In the investigations reported here browning was measured by increase in Klett photoelectric colorimeter reading with No. 42 filter. The latter was chosen because, although the browned orange juice and solutions exhibit general absorption increasing toward the shorter wave length, optical density in the region of 400-500 mμ is more frequently used than at other wave lengths.

RESULTS AND DISCUSSION

Chromatographic separations. In our previously reported investigations, no discernible changes in amino acids were detected after storage for 4 months even though the ascorbic acid had disappeared and the juice had reached its maximum in browning *(12)*. A sample of Navel orange juice stored for one year, however, which was examined was noticeably different from freshly extracted Navel orange juice. That differences in the chromatographically separable constituents occurred after long storage is shown in Figures 3 and 4. Figure 3 shows the paper chromatograms obtained

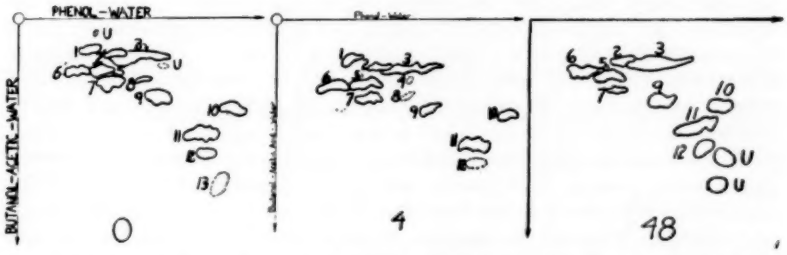

Figure 3. Amino acids occurring in strained Valencia orange juice after storage exposed to air for 0, 4 and 48 months. The identified ninhydrin positive spots are: 1—lysine, 2—asparagine, 3—arginine, 4—glutamine, 5—serine, 6—aspartic acid, 7—glutamic acid, 8—threonine, 9—alanine, 10—proline, 11—gamma amino butyric acid and 12—valine and methionine.

with strained Valencia orange juice initially and after storage for 4 and 48 months. Since these were prepared using the same aliquots of alcohol extract under similar conditions it is possible to draw the following conclusions as to changes that occurred after 4 years' storage: (1) lysine apparently disappeared although it may be included in the asparagine spot; (2) glutamic acid content as observed by visual comparison with previous spots has decreased; (3) a new orange colored spot occurred together with serine; (4) alanine, arginine, asparagine, gamma amino butyric acid and proline have not decreased appreciably; (5) valine and possibly methionine are again present but two new spots occur corresponding to the pheynl alanine and leucine group. Figure 4 shows similar comparison for

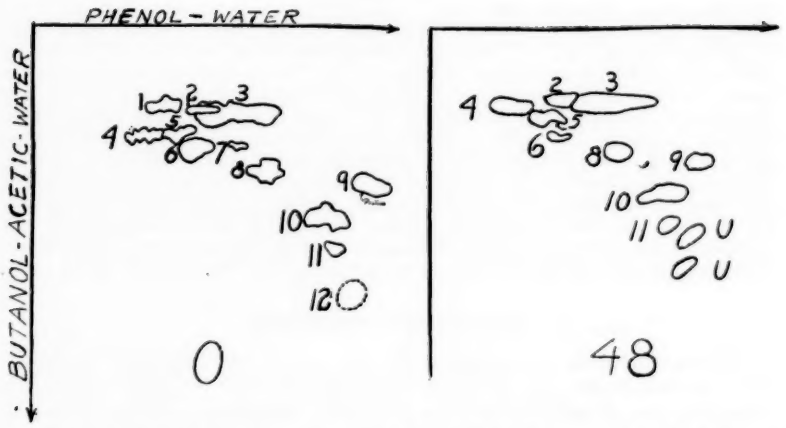

Figure 4. Amino acids occurring in centrifuged Valencia orange juice initially and after storage for 4 years exposed to air at room temperature. The identifiable ninhydrin positive spots are: 1—lysine, 2—asparagine, 3—arginine, 4—glutamic acid, 5—serine, 6—aspartic acid, 7—glutamic acid, 8—threonine, 9—alanine, 10—proline, 11—gamma amino butyric acid and 12—valine and/or methionine.

the centrifuged juice and here again there is evidence that: (1) lysine has disappeared; (2) glutamic acid content has decreased appreciably; (3) a new orange colored spot is present together with serine; (4) little appreciable change occurred in other amino acids; and (5) two spots corresponding roughly to positions that would be occupied by phenyl alanine and leucine appear.

These results, though qualitative and incomplete, indicate that of the amino acids present in Valencia orange juice, lysine and glutamic acid apparently are more reactive but even these react more slowly than would be expected. Whether the decrease in these amino acids, in the amounts observed, is due to their reaction with constituents such as sugars or to Strecker degradation *(1-3, 38)* is not known. However some evidence for the former was found by chromatographing aliquots of the browned juices after concentration *in vacuo*. The occurrence of ninhydrin positive spots corresponding in mobility to the glucose-glycine compounds reported by Chichester *et al. (7)* was found, but these were present in relatively very small concentrations.

Some evidence for ninhydrin positive constituents other than amino acids was found also in the ascorbic acid-alanine solution, in the ascorbic acid-lysine solution, and in the ascorbic acid-dextrose-lysine solution. This was much more definite in the oxidized ascorbic acid-glycine solution. No evidence of the presence of similar amino acid compounds was found in oxidized ascorbic acid-lysine and in oxidized ascorbic acid-fructose-lysine solution. These were chromatographed after the respective solutions had become considerably browned.

Fractionation of the orange juice constituents involved in browning. The browning of filtered Navel orange juice and of the fractions obtained

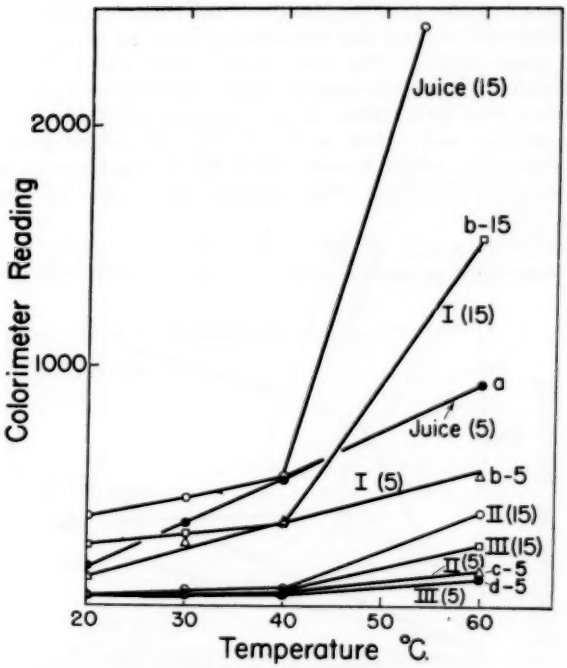

Figure 5. Temperature dependence of the browning of orange juice and fractions obtained from it, two stages (after 5 and 15 days). Fraction I—juice less cation absorbable constituents; II—juice less anion absorbable constituents, III—juice less cation and anion absorbable constituents.

from it by resin treatment is shown in Figures 5 and 6. In Figure 5 the browning observed after 5 and 15 days storage at the several storage temperatures tested is plotted against temperature. The high temperature dependence of the browning reaction in orange juice *(21)*, dried apricots *(41)*, and other products is well established *(17, 40)* but observations on the temperature dependence of the reactions involved at all stages of browning are very limited. It is usually assumed that, in the temperature range of 20-60° C., the reactions are similar and temperature dependence is linear. Thus, for concentrated orange juice, Curl *(8)* reported the rate of browning to be about 20 times as fast at 49° C. as at 27° C. Our data summarized in Figure 5 shows that in the initial stages of browning, the intensity of color change is linear over the temperature range 20-60° C. but in later stages marked differences occur between 40 and 60° C. At the end of 15 days' storage, the samples of juice had to be diluted (1:4) and that of fraction I (1:2) in order to read the color values on the Klett colorimeter scale. For straight juice the color reading calculated at 60° was 3350 in comparison with 540 at 40° C. Similar breaks occurred in the other fractions. The browning of straight orange juice is more intense and

increases more rapidly with temperature than that of juice treated with cation exchange resins (and this presumably lower in metallic cations and nitrogenous constituents). The juice treated with anion exchange resins browned initially only slightly even at higher temperatures and was similar to juice treated with both resins. Acidic constituents other than citric acid (probably ascorbic acid) thus accounted for the major portion of the browning. Only after longer storage and at 60° C. was there an appreciable difference between the anion resin treated juice and that treated with both resins.

The rate of browning at 20° C. and 40° C. is shown in Figure 6. Whereas browning of untreated juice increased linearly with time at room

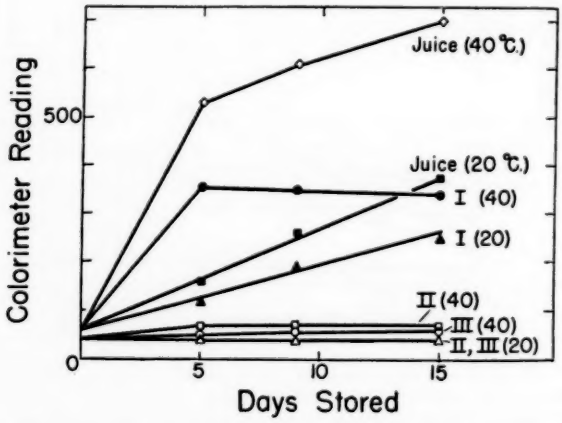

Figure 6. Comparison of course of browning in Navel orange juice at 20 and 40° C. (I—cation resin treated; II—anion resin treated; III—both resins treated).

temperature, at 40° C. the rate was more rapid at first and then decreased. This was also true for the cation resin treated juice. Although the removal of part of the cationic constituents decreased the initial rate of browning at both 20 and 40° C. slightly it appreciably affected the course of browning in later stages.

Ascorbic acid–amino acid–sugar systems. Since the chromatographic observations reported above indicated that of the many amino acids present in orange juice, lysine, and glutamic acids were more likely to be involved in browning, the contribution of these amino acids in comparison with alanine, asparagine, and glycine was investigated in model systems. At 40° C. in the presence of air ascorbic acid, at the concentration level of 0.025 M (440 mg./100 ml.) used, browned more rapidly alone initially than in combination with sugars and/or amino acids. As shown in Figure 7 and Table 1, the addition of dextrose decreased the rate of browning. The addition of amino acids other than alanine or glycine appreciably decreased the rate and extent of browning initially, particularly so for glutamic acid. In later stages, in the presence of added amino acids, particularly lysine, the

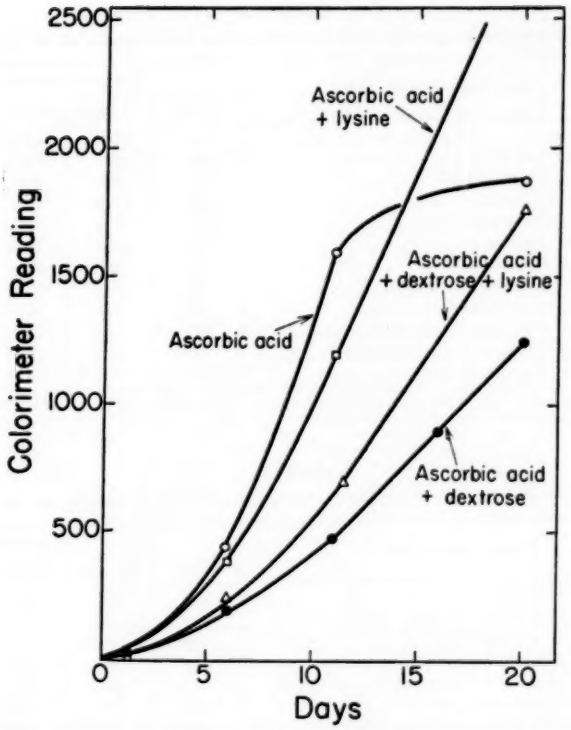

Figure 7. Relative rates of browning of ascorbic acid with and without added dextrose and lysine.

rate and extent of browning was increased. The inhibiting affect of dextrose was overcome by addition of all amino acids except glutamic acid and markedly so by lysine. No detectible browning occurred in the citrate buffer containing dextrose, fructose, and amino acids alone or in combination.

Dehydroascorbic acid (?)–sugar–amino acid system. In solutions containing carbon treated ascorbic acid, as shown in Table 2 and Figure 8, browning occurred somewhat more rapidly at first but the extent of browning was considerably less. This may have been due to losses in reactive oxidized ascorbic acid solution by adsorption on carbon or to other factors. Browning reached its maximum in 6 days and then increased slightly if at all. In this system, however, the addition of dextrose and fructose also decreased rate and extent of browning but the addition of amino acids markedly increased both rate and extent of browning. Of the amino acids tested lysine was most reactive.

Effect of ascorbic acid concentration. Since ascorbic acid appears to be the most reactive constituent in the systems investigated, the effect of varying concentrations of ascorbic acid from 44 mg. to 1100 mg. per 100 ml. on rate and extent of browning was determined. The extent of brown-

TABLE 1

Browning in ascorbic acid-dextrose-amino acid systems at 40° C.

Constituents present	Colorimeter reading[1]			
	6 days	11 days	20 days	45 days
Ascorbic acid	440	1600	1920	2165
Ascorbic acid + alanine	520	1800	1840	3150
Ascorbic acid + asparagine	410	1360	1830	2450
Ascorbic acid + glycine	450	1200	2130	3500
Ascorbic acid + glutamic acid	185	610	2175	2700
Ascorbic acid + lysine	385	1200	2880	3300
Ascorbic acid + dextrose	190	480	1350	1500
Ascorbic acid + dextrose + alanine	170	420	1140	1500
Ascorbic acid + dextrose + asparagine	166	460	1215	2100
Ascorbic acid + dextrose + glycine	189	485	1290	2900
Ascorbic acid + dextrose + glutamic acid	96	272	930	1550
Ascorbic acid + dextrose + lysine	240	640	1770	2550

[1] Readings over 600 were obtained by dilution of the samples with distilled water and calculated on the assumption that Beer's law applied.

TABLE 2

Browning in dehydroascorbic acid-sugar-amino acid systems at 40° C.

Constituents present	Colorimeter reading			
	6 days	11 days	20 days	45 days
Dehydroascorbic acid	100	110	108	124
Dehydroascorbic acid + glucose	84	71	68	76
Dehydroascorbic acid + fructose	69	64	61	72
Dehydroascorbic acid + alanine	129	165	165	220
Dehydroascorbic acid + asparagine	165	193	193	240
Dehydroascorbic acid + glycine	163	193	195	254
Dehydroascorbic acid + glutamic acid	147	170	170	206
Dehydroascorbic acid + lysine	187	230	236	296
Dehydroascorbic acid + glucose + alanine	73	75	73	84
Dehydroascorbic acid + glucose + asparagine	61	62	59	70
Dehydroascorbic acid + glucose + glycine	61	62	60	70
Dehydroascorbic acid + glucose + glutamic acid	60	60	57	65
Dehydroascorbic acid + glucose + lysine	70	72	65	74
Dehydroascorbic acid + fructose + alanine	81	75	73	82
Dehydroascorbic acid + fructose + asparagine	72	69	68	90
Dehydroascorbic acid + fructose + glycine	79	78	75	101
Dehydroascorbic acid + fructose + glutamic acid	65	57	54	68
Dehydroascorbic acid + fructose + lysine	95	100	100	143

ing in the initial stages of the reaction is shown in Figure 9. This indicates that browning is at a maxmium at concentrations of 100-200 mg. per liter but shifts from 132 mg. per cent to 220 mg. as period of storage increased. Up to this maximum, the extent of browning increases linearly with increases in ascorbic acid content and then decreases. The extent of browning of ascorbic acid solutions, however, is not directly related to initial ascorbic acid concentration as shown in Table 3. The relative colorimeter reading does not increase in proportion to increase in ascorbic acid content. At levels of 44 to 220 mg. per 100 ml. it is less than directly proportional at first and then is greater; at levels of 330 mg. per 100 ml. and higher it is

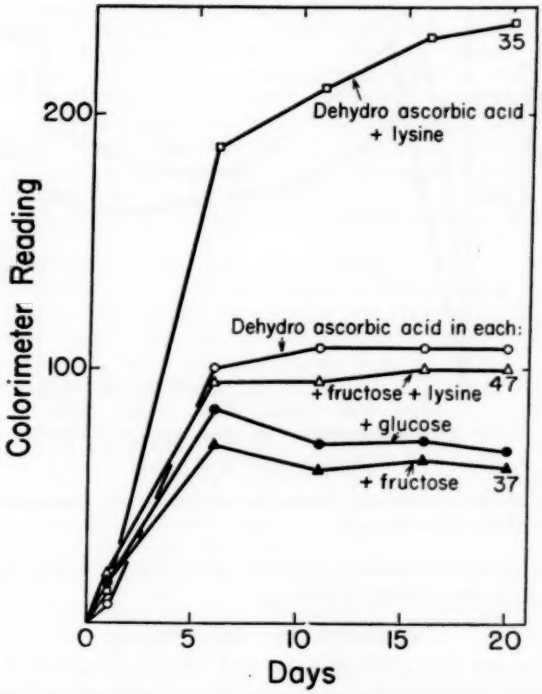

Figure 8. Rate of browning of dehydroascorbic acid systems at 40° C.

always less, although it increases with increase in storage. The decrease probably is due to increase in reducing level of the solution with increase in ascorbic acid content. If this explanation is correct, then the oxidation-reduction potential should have a more important role in browning than

TABLE 3

Effect of ascorbic acid concentration on rate of browning of ascorbic acid–citric acid solutions stored exposed at 40° C.

Ascorbic acid concentration			Relative colorimeter reading				
mg./100 ml.	M	Relative	Days stored				
			1	4	6	10	30
44	0.0025	1	1.00	1.00	1.00	1.00	1.00
88	0.0050	2	1.02	1.82	2.25	2.27	2.24
132	0.0075	3	1.07	1.38	2.87	3.67	3.07
176	0.010	4	1.00	1.26	2.54	4.82	4.03
220	0.0125	5	1.00	1.15	2.25	5.53	4.69
330	0.01875	7.5	0.80	1.04	1.99	5.44	6.40
440	0.025	10	0.80	1.00	1.83	4.88	9.92
660	0.0375	15	0.60	0.87	1.56	3.85	11.03
1100	0.0625	25	0.57	0.90	1.64	4.32	19.50

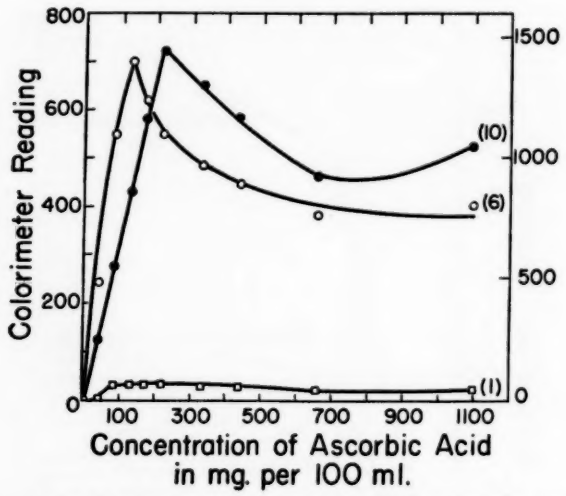

Figure 9. Extent of browning of ascorbic acid at various concentrations after 1, 6 and 10 days storage at 40° C.

the concentration of oxidized ascorbic acid present. This effect is being investigated in more detail at present.

SUMMARY AND CONCLUSIONS

The extensive data published on the browning of foods in general and particularly on the browning of orange juice is reviewed and some apparent discrepancies are explained.

The apparent decrease in lysine and glutamic acid occurring in later stages of browning of orange juice stored in presence of air for 4 years at room temperature and some evidence for the presence of ninhydrin positive amino acid compounds is reported.

New data on the oxidative browning of ascorbic acid–amino acid–sugar system is presented which indicates that ascorbic acid is the most reactive component and that glucose and fructose inhibit its browning. Amino acids in the initial stages inhibit browning, but in later stages increase it. The concentration of ascorbic acid initially present has a marked effect on rate and extent of browning of ascorbic acid solutions.

Acknowledgments

The chromatograms of the four-year-old juice samples were prepared and interpreted by P. M. Townsley so that direct comparison with his previously reported data could be made. Chromatographic detection of possible presence of amino acid compounds was made by Dr. C. O. Chichester. Dr. Guido De Luca analyzed the sediment for uronide content and R. Peterson and E. Zuegg assisted in making some of the colorimetric measurements reported.

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A RE-EVALUATION OF BIOLOGICAL POTENCY OF BETA CAROTENE[a]

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The International Unit of vitamin A activity was defined in 1934 as the biological response given by 0.6 μg. of pure beta carotene. Subsequently, the United States Pharmacopoeia prepared and distributed several standards consisting of cod liver oil preparations to which were assigned certain biological potencies. These preparations, however, did not prove to be suitable and their biological potency in terms of beta carotene was apparently greatly over-rated. With the isolation and availability of pure vitamin A esters, a new standard was adopted by the United States Pharmacopoeia consisting of a solution of vitamin A acetate in cottonseed oil. On the basis of the vitamin A content of this preparation, 0.3 μg. gives one unit of vitamin A activity. This new unit of vitamin A was presumed to be identical to the old unit based on 0.6 μg. of carotene. If such were the case, it would appear that the organism is able to recover only one molecule of vitamin A from one molecule of carotene.

During the past few years, several publications have appeared *(2, 3, 17, 20)* indicating that under the proper conditions, all-trans beta carotene gives a biological response approaching that given by an equal weight of vitamin A. This agreed with the fact that biological assays on our commercial carotene products derived from carrots were higher than could be explained on the basis of their carotene content.

At the present time, most of the carotene being manufactured is used in margarine where its biological activity is included as a part of the vitamin A potency. Inasmuch as the U.S.P. biological assay is the only officially accepted method for the determination of the vitamin A activity of margarine, it is obviously of great importance to know the biological activity of carotene in terms of this assay method.

EXPERIMENTAL

With but one exception, all of the assays reported here were on crystalline carotene products derived from carrots. Many of the samples were commercial materials which contained some alpha carotene along with the beta carotene.

The relative amounts of the two carotenes were determined by chromatographic separation on columns of magnesium oxide (Westvaco No. 2642) : Hi-Flo Super Cel or of calcium hydroxide followed by spectrophotometric assays. The concentration of beta carotene was calculated by measurement of the absorbance at 450 mμ in hexane solution and use of an extinction coefficient ($E^{1\%}_{1cm.}$) of 2520. Alpha carotene was measured at 446 mμ using an extinction coefficient of 2720. In addition, the absorbance ratio

[a] Presented at the Sixteenth Annual Meeting of the Institute of Food Technologists, St. Louis, June 11, 1956.

[b] Deceased February 20, 1956.

340/450 was determined to establish the absence of stereoisomers. It is considered that when this ratio is less than 0.06, the carotene is substantially all-trans.

All of the biological assays reported here were performed either at the University of Southern California by Dr. Harry J. Deuel, Jr., or at the Food Research Laboratories under the direction of Dr. Bernard L. Oser. All were carried out using the official U.S.P. rat growth method against the reference vitamin A acetate standard.

Tests at the University of Southern California were carried out by the U.S.P. XIII method wherein the supplements were fed 6 times weekly. Samples were dissolved in cottonseed oil containing 0.5% alpha tocopherol and were fed in 0.1 ml. doses by syringe. The reference vitamin A acetate standard was fed at 1.25 units per day for the single level assays and at 1 and 2 units per day for the multiple level assays. The feeding dilutions were all prepared at the start of the assay and stored in ¼-ounce brown bottles under nitrogen in the freezer until used. Fresh samples were opened every 4 days.

Samples tested by the Food Research Laboratories were all assayed by the U.S.P. XIV method. The supplements were fed on alternate days by syringe in 0.1 ml. doses containing 2.0 and 2.828 units. Samples and standards were diluted for feeding with cottonseed oil containing 0.3% mixed tocopherols. The feeding dilutions were subdivided into 4 ml. screw-cap vials, flushed with nitrogen, sealed and stored at –5° C. until required for feeding. A fresh vial was thawed and opened for each feeding.

In comparing the biological assays with the spectrophotometric determinations, the "spectrophotometric potency" was calculated on the assumption that 0.6 μg. of beta carotene or 1.2 μg. of alpha carotene were equivalent to 1 unit of vitamin A activity.

RESULTS AND DISCUSSION

Results of these tests carried out at the University of Southern California are summarized in Table 1. All of the samples tested were made from commercial carotene crystals as derived from carrots. Sample "D" is of particular interest inasmuch as it was analyzed as part of a collaborative assay on physical methods of carotene assay. Twelve laboratories in various sections of the country participated in this study and reported spectrophotometric and chromatographic assays on these crystals. The figures given in Table 1 for this sample are the average of the assays reported by these laboratories.

Since samples A and B were assayed at only one level, it is impossible to assign an actual potency to them other than to say that the biological activity was greater than the assumed potency. However, as can be seen from Table 3, the average weight gain of the rats fed the carotene samples was considerably higher than that of the rats fed the reference vitamin A acetate at the same assumed potency which, in turn, was appreciably higher than the "spectrophotometric potency."

Samples C and D, however, were fed at three assay levels with two reference vitamin A acetate levels. Under these conditions, it is possible to obtain a very close estimate of the actual biological potency of the material being tested. As can be seen in Table 1, these potencies are about one-third higher than can be explained on the basis of the spectrophotometric assay.

The assays carried out at Food Research Laboratories are summarized in Table 2. The chromatographic and spectrophotometric assays shown are those reported by them. However, it should be stated that they use an extinction coefficient of 2590 for calculating the concentration of beta carotene rather than 2520 such as is used in our laboratory. All of the biological

TABLE 1

Analyses of natural carotene samples assayed by U.S.P. XIII procedure

Sample	Description	Physical assay				Biological assay			
		Total pigment calculated as beta carotene %	Alpha carotene %	Beta carotene %	Spectrophotometric potency units per gram	Number of assay levels	Assumed potency USP per gram	USP units per gram	Per cent of "spectrophotometric potency"
A	Carotene butter color	0.24%	0.03% [1]	0.20% [1]	3,570	1	4,000	> 4,000	> 112%
B	Microcrystalline carotene in oil	24.2%	4.3% [1]	18.7% [1]	347,000	1	400,000	> 400,000	> 115
C	Microcrystalline carotene in oil	24.40%	4.72%	18.8%	353,500	3	400,000	455,000	129
D	Commercial carotene crystals [2]	90.6%	19.5%	69.6%	1,323,000	3	1,600,000	1,820,000	138

[1] The distribution of alpha and beta carotene in these samples was estimated.
[2] Collaborative assay sample. See text.

TABLE 2

Analyses of natural carotene samples assayed by U.S.P. XIV procedure

Sample	Description	Physical assay				Biological assay		
		Total pigment calculated as beta carotene %	Alpha carotene %	Beta carotene[1] %	Spectrophotometric potency units per gram	Assumed potency USP per gram	USP units per gram	Per cent of "spectrophotometric potency"
E	Alpha carotene	99.8%[2]	97.7%	0	814,000	1,150,000	1,145,000	141%
F	Beta carotene	99.2%	.....	.....	1,653,000	2,300,000	2,386,000	144
G	Commercial carotene crystals	86.3%	19.0%	64.0%	1,225,000	1,300,000	1,494,000	122
H	Microcrystalline carotene in oil	23.4%	6.3%	15.9%	317,500	465,000	450,300	142
I	Microcrystalline carotene in oil	23.3%	5.3%	16.4%	317,400	450,000	365,000	115
J	Microcrystalline carotene in oil	22.5%	6.2%	15.0%	301,660	420,000	480,000	159

[1] Calculated using an $E^{1\%}_{1cm.}$ of 2590 at 452 mμ.

[2] Calculated as alpha carotene.

tests were carried out with two reference levels and two assay levels. Samples E and F were high purity alpha and beta carotene made by chromatographic separation of the commercial carotene crystals and crystallization from hexane. It will be noted that 0.87 μg. of alpha carotene and 0.42 μg. of the beta carotene gave a biological activity equivalent to one U.S.P. vitamin A unit.

It will be seen that these assays confirm the greater than theoretical activity of carotene as found in Table 1. On the basis of these tests, the assumption made in computing the spectrophotometric potencies greatly underestimated the true biological activity of both alpha and beta carotene.

At the same time as samples E, F, G and H were run, the International Reference Standard for provitamin A was tested. This material is a solution of carotene in vegetable oil containing 200 units per gram *(24)*. It was fed to two groups at an assumed potency of 200 units per gram. The assayed potency was 194 ± 13 U.S.P. units per gram. Thus of all the carotene samples tested in that series, it was the only one which did not show a higher than theoretical potency.

In view of the publication by Deuel, Greenberg, Savage and Melnick *(4)* regarding the augmented biological activity of carotene when fed in margarine, sample H was also fed to two levels of rats using margarine oil[c] (to which the 0.3% tocopherols were added) as a carrier. This particular margarine oil contained all the oil-soluble additives normally used in margarine with the exception of the vitamin A. It was assayed concurrently with sample H and gave a biological activity of 486,000 units per gram compared to the 450,300 when this sample was fed in cottonseed oil. Although the increase in biological potency is not as striking as that reported by Deuel *et al.*, it indicates that margarine oil may enhance the biological activity of carotene.

In attempting to explain the discrepancy between the estimated potencies and the actual activity, a study was made of the published data on the reported biological activity of carotene as against vitamin A. Particular emphasis was placed on evaluating the quality of the materials used in the published studies. This is necessary inasmuch as a considerable amount of work has been published based on materials of very doubtful purity. This made it necessary, for example, to disregard work wherein carotene was assayed against the U.S.P. Reference Cod Liver Oil preparations. With the availability of crystalline vitamin A esters of high purity along with the development of more precise spectrophotometric techniques, this problem has become less acute in the case of the preformed vitamin A. However, in the case of carotene, a number of research workers have continued to use so-called pure carotene preparations despite the warnings by Guerrant, Chilcote, Ellenberger, and Dutcher *(8)* and Koehn *(17)* in 1948 that many of these contained large amounts of impurities. Even more suspect are publications wherein liquid "carotene concentrates" are used and there is a likelihood of the presence of cis-trans isomers along with other carotenoids.

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[c] We gratefully acknowledge the kindness of The Best Foods, Inc. in supplying this material.

TABLE 3
Gains in weight of rats in biological assays

Material fed	Number of rats	Daily dose per rat	Average initial weight	Average weight gain 28 days
		Micrograms	*Grams*	*Grams*
Assay 1.				
Sample A	13	312.5	93.2	41.2 ± 2.0[1]
U.S.P. reference standard	16	125	92.8	35.0 ± 2.9
Assay 2.				
Sample B	15	3.125	94.2	38.3 ± 2.9
U.S.P. reference standard	15	125	96.2	35.5 ± 1.7
Assay 3.				
Sample C	12	3.13	106.8	42.3 ± 4.5
	12	4.28	107.6	54.2 ± 4.0
	11	5.63	108.0	66.2 ± 2.8
U.S.P. reference standard	11	125	103.9	36.3 ± 2.4
	11	225	102.5	60.4 ± 5.8
Assay 4.				
Sample D	13	0.6	109.9	41.5 ± 5.0
	13	0.9	110.3	58.4 ± 3.3
	15	1.2	107.3	66.9 ± 3.7
U.S.P. reference standard	14	100	107.0	39.0 ± 3.3
	14	200	104.4	64.3 ± 3.3
Assay 5.				
Sample E	8	0.87	116.3	31.6 ± 5.4
	8	1.41	112.6	50.0 ± 7.4
Sample F	8	0.627	113.8	56.4 ± 3.6
	8	0.87	115.1	69.3 ± 3.7
Sample G	8	0.79	122.5	39.3 ± 3.3
	8	1.11	115.6	60.0 ± 6.5
Sample H	8	3.04	120.6	48.3 ± 6.3
	8	4.30	117.0	69.8 ± 6.8
Sample H (fed in margarine oil)	8	3.04	121.9	54.0 ± 6.5
	8	4.30	116.4	73.1 ± 3.7
International Provitamin A Standard (200 units per gram)	8	7,070	117.4	47.9 ± 2.1
	8	10,000	118.8	70.3 ± 5.6
U.S.P. reference standard	8	141.4	114.5	50.3 ± 4.4
	8	200	114.5	71.8 ± 3.8
Assay 6.				
Sample I	8	2.67	118.1	40.5 ± 5.1
	8	4.00	115.9	54.8 ± 3.4
U.S.P. reference standard	8	100	116.8	38.9 ± 4.6
	8	200	117.3	73.5 ± 5.3
Assay 7.				
Sample J	10	3.37	112.0	53.7 ± 4.4
	10	4.76	111.1	66.3 ± 5.7
U.S.P. reference standard	10	141	109.3	46.7 ± 2.5
	10	200	104.03	62.4 ± 2.8

[1] Standard deviation of mean. We thank Mr. Carl A. Taylor of this laboratory for his help in making these calculations.

It should be emphasized that the literature survey was aimed primarily at studies using rat growth as a criterion for vitamin A activity such as is specified in the U.S.P. biological assay. Thus no attempt is made here to cover the large amount of work involving liver-storage, etc.

The concept that 0.3 μg. of vitamin A alcohol has the same biological activity in the rat growth test as does 0.6 μg. of beta carotene is founded largely on two collaborative assays of pure vitamin A ester preparations against the International Reference beta carotene *(15)*. The first of these was conducted in Great Britain using crystalline vitamin A beta naphthoate and the potency of vitamin A alcohol was found to be 3,200,000 units against a defined potency of 1,666,666 for the International Reference beta carotene standard *(19)*. Unfortunately, no spectrophotometric data were given for the beta carotene standard although it was given for the vitamin A ester.

The second collaborative assay was conducted in 1946-47 under the sponsorship of the United States Pharmacopoeia[d] with 11 laboratories participating *(21)*. In this assay, a solution of crystalline vitamin A acetate in oil ($E^{1\%}_{1\ cm.}$ @ 325 mμ on the unsaponifiable fraction = 5.279) was compared with an oil solution of the International Reference carotene dissolved in cottonseed oil whose potency was estimated to be 250 units per gram on the basis of spectrophotometric assay. The average biological potency of the vitamin A acetate solution was found to be 9,735 units per gram. Subsequently, a new lot of the vitamin A acetate solution was made up whose extinction coefficient of 5,442 was slightly higher than the first. This was distributed as a standard solution containing 10,000 U.S.P. vitamin A units per gram. Later, the World Health Organization defined the unit of vitamin A activity as that given by 0.344 μg. of the standard preparation of vitamin A acetate which is equivalent to 0.3 μg. of vitamin A alcohol *(24)*.

These figures for the biological activity of vitamin A in terms of carotene also coincide closely with the 3,000,000 units per gram obtained for the crystalline vitamin A alcohol (with methanol of crystallization) first isolated by Holmes and Corbet *(12)* and assayed against the International Reference Carotene standard. Although considerably higher potencies were reported by Baxter and Robeson for crystalline aliphatic esters of vitamin A *(1)*, these were based on the old Reference Cod Liver Oil standard.

Inasmuch as the above cited studies were unquestionably carried out quite carefully, any argument against the validity of the 2:1 ratio of biological activity for vitamin A against carotene (on a weight for weight basis) must rest on one or both of the following premises: (a) The International Reference carotene preparation did not consist of pure trans beta carotene or (b) the technique used may have favored the utilization of one form of vitamin A over the other. It would appear that both of these may be partially correct.

Shortly after the first collaborative assay on vitamin A beta naphthoate was published, two papers appeared indicating that the International Reference carotene was impure. Hunter and Scott *(16)* prepared some alpha and beta carotene of high purity and these samples were assayed by Wilkinson *(23)* against the reference beta cartoene standard, using the rat growth method. Their beta carotene demonstrated 20% greater activity than did

[d] We acknowledge with thanks the kindness of Dr. Adley B. Nichols of the U.S.P. in allowing us to examine the letters describing these assays.

the International Standard. Subsequently, Van Eckelen *(22)* reported chromatographic studies on the International Standard carotene wherein he found the total carotene to range from 75-92% and the beta carotene from 66-84%.

Inasmuch as this supply of the International Reference beta carotene was exhausted in 1944, a fresh lot was prepared which reportedly had the same spectral absorption as did the preceding material *(15)*. This material was used by Guerrant at Pennsylvania State College to prepare the solutions used for the U.S.P. collaborative assay. In reporting the results of their tests wherein the U.S.P. Reference vitamin A acetate was assayed biologically against the International Standard beta carotene, Ellenberger, Guerrant, and Chilcote *(5)* noted that while the vitamin A acetate was quite pure, the beta carotene had an extinction coefficient of only 2300 in hexane. Thus on the basis of the commercial extinction coefficient of 2520, this material was at best 91.2% pure. In terms of this carotene, pure vitamin A acetate supplied 2,930,000 units per gram. In these bioassays, no tocopherols were added other than those supplied by the 5% hydrogenated oil (Crisco) in the diet. The carotene supplements were first dissolved in benzene and then diluted with oil without removal of the solvent. This resulted in benzene concentrations in the supplements as fed of as high as 0.77%. In view of the sensitivity of the rat growth assay to foreign chemicals *(13, 22)*, this small amount of benzene may interfere with full utilization of the carotene.

It appears, therefore, that the International Reference provitamin A standard which has been used to establish the potency of the present vitamin A acetate standard for vitamin A activity may not be a true measure of the biological potency of beta carotene. The bioassay reported here on this material confirms the potency established in the U.S.P. Collaborative Assay but the material shows considerably less activity than did carotene samples of known composition.

The relative responses of carotene and vitamin A in the U.S.P. assay method are also complicated by their different response to accessory factors in the diet. The importance of adequate tocopherols in the diet has been demonstrated by Hickman *(10)*, Lemley *et al. (18)* and many others. In the U.S.P. diet, the tocopherols are furnished chiefly by the 5% cottonseed oil in the diet. In general, it appears that carotene requires more tocopherol to produce optimum growth response than does vitamin A. Therefore, if there is an insufficiency of tocopherols, vitamin A will tend to give a better growth response than carotene.

As yet, the United States Pharmacopoeia has not taken official cognizance of the necessity of adding supplementary tocopherols to the diet. However, it has in fact become common practice to control this variable in biological assays by supplementing with tocopherols the cottonseed oil used in diluting the samples and standards *(4)*.

Vitamin B_{12} appears to be another nutritional factor that is important in vitamin A assays. High and Wilson *(11)* demonstrated that the U.S.P. diet is deficient and that its inclusion results in better growth response to feedings of carotene and vitamin A. As with tocopherols, carotene appears to be more sensitive to a B_{12} deficiency than is preformed vitamin A.

By using a modified U.S.P. diet and feeding adequate amounts of tocopherol, Sherman *(20)* found that carotene gave a considerably higher biological activity than did an equal unitage of vitamin A. The purity of both the carotene and the vitamin A was carefully established.

Koehn *(17)* fed spectroscopically pure vitamin A and beta carotene to rats on a semi-synthetic diet which differed significantly from that in the United States Pharmacopoeia. Under these conditions, vitamin A and beta carotene gave similar growth response on a weight for weight basis rather than a mole for mole. This work was confirmed by Burns, Hauge, and Quackenbush *(2, 3)* who found that in the levels fed 1 mg. of tocopherol daily, vitamin A and carotene gave substantially the same growth response. However, with 2 mg. of tocopherol daily, the utilization of beta carotene was diminished significantly.

Hove *(14)* noted that the most obvious deviation from the U.S.P. diet in the above cited work was the omission of yeast from the diet. On repeated bioassay, it was found that without yeast beta carotene had 70-90% of the activity of vitamin A when 1 mg. of alpha tocopherol was also fed. On inclusion of yeast in the diet, vitamin A gave a *greater* growth response while beta carotene gave a *lesser* response.

Deuel *et al. (4)* attributed the 30% higher than theoretical activity of carotene in margarine oil to the synergistic action of the lecithin and mono- and diglycerides plus the natural stability of carotene in this carrier. While at that time it was thought that lecithin enhanced the biological efficacy of vitamin A and carotene *(6)*, it was since shown by Guerrant and Thompson *(9)* that the effect was due to a carotene-like impurity in lecithin and that the effect was additive rather than synergistic. In view of the results reported here, it appears more likely that the effect noted by Deuel *et al.* was due to both the higher than theoretical activity of beta carotene along with some increase in response by feeding in margarine oil.

SUMMARY

The biological assays reported here indicate that pure all-trans beta carotene has a biological activity of 2,200,000 to 2,500,000 U.S.P. units per gram in terms of the U.S.P. reference vitamin A acetate standard. It therefore seems very doubtful that carotene splits in such a manner as to give only one molecule of vitamin A.

On the basis of these results as well as previously published data, there appears to be reason to question the purity of the International Provitamin A preparation or that it is indicative of the biological activity of pure trans beta carotene.

Accordingly, it is believed that a re-evaluation of the accepted vitamin A potency of beta carotene is warranted.

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STUDIES ON THE CHANGES IN THE WATER SOLUBLE CARBOHYDRATES OF CHAPATIES[a] DURING AGING

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In connection with our studies on longer keeping chapaties, it was found desirable to study the changes that occur in the water soluble saccharides of chapaties during storage. It was thought that such data would be helpful in evaluating staling phenomenon in chapaties and in finding out whether these changes would in any way be comparable to those taking place in bread *(1, 2, 3, 7, 8, 11)*.

Our present investigation concerns changes in water soluble carbohydrates of chapaties stored under the following conditions: (a) packaged in a water-impervious plastic bag and stored at room temperature (28° C.) and at refrigerator temperature (8° C.) for a period of 72 hours; (b) packaged in accordance with the method developed at this laboratory for making longer keeping chapaties, and stored at room temperature for about 6 months; and (c) wrapping 10 chapaties together with a piece of cloth and storing at room temperature. The last-mentioned practice is very common in India, especially among the poorer people, who keep chapaties overnight for consumption during the next day.

To secure specific information about the relative changes in the various types of saccharides of chapaties during storage, it was thought advisable to include in this study a brief account of the work carried out on this aspect, using circular paper chromatographic technique. For purposes of comparison a study has also been made of the relative changes in the saccharides of whole wheat flour on storage.

METHOD

Chapaties were prepared from freshly ground whole-wheat flour after mixing it with water and kneading it to a dough of suitable consistency (3 parts of flour and 2 parts of water by weight). After kneading, it was allowed to stand for ½ hour. A weighed amount of dough (55 g.) was taken, rolled to a circular disc 5 inches in diameter and about 2 mm. in thickness, and baked on a hot plate. During baking, every effort was made to keep the various factors like time and temperature of baking, width and thickness of chapaties, etc., constant for different batches.

After baking, one chapati was immediately taken for analysis and the other stored, according to the conditions mentioned above, for further experiments.

Water soluble extracts of chapaties were prepared by taking a 40-g. portion of a chapati, cutting it into small pieces, and after soaking these in water for about 10 minutes with occasional stirring, macerating them in a Waring blender to form a uniform slurry. The slurry was transferred to a 250 ml. flask and volume made to the mark with water. After thoroughly mixing, the contents were centrifuged and filtered. The filtrate was used for analysis of the various constituents.

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[a] Chapati: Indian equivalent of bread prepared by rolling dough made from whole wheat flour to a disc of about 2 mm. in thickness and 4-5 inches in diameter, and baking on a hot plate.

Water soluble solids and water soluble reducing sugars before and after acid hydrolysis were determined by the method used by Jackel, Schaeder and Schultz *(7)*.

Moisture content of chapati was determined by weighing a representative sample, cutting it into small pieces and drying to constant weight.

Chromatographic analysis was carried out by the modified circular paper technique of Giri and Nigam *(5)* using butanol: acetic acid: water (40:10:50) as the irrigating medium and diphenyl-amine-aniline phosphoric acid as the developer. Material for chromatography was prepared by extracting a given weight of the material with 50% alcohol, concentrating it in a water bath to 1/5 of its original volume, and centrifuging the extract to clarify it. The chromatograms were run overnight for 18 hours.

RESULTS AND DISCUSSION

1. Quantitative studies on the changes in the water soluble carbohydrates of chapaties. Changes in the water soluble starch, soluble reducing sugars, and soluble solids of chapaties during storage at room and refrigerator temperatures are indicated in Table 1. The data show that the conditions of storage have definite effects on the amount of the various water soluble constituents of chapaties. Within 24 hours of aging, there is a decrease in the water extractable materials, this being more pronounced at lower temperature. As can be seen from Table 1, at 8° C. there is a 19.6% decrease of the soluble solids whereas at room temperature the corresponding decrease is only 11.2%. However, after this initial decrease, observed during the first 24 hours, there is a progressive increase in the total solids, so that after 72 hours of aging at room temperature the soluble solids content exceeds the original level.

Reducing sugars content of chapaties also changes on aging. There is a decrease only when the storage temperature is low, the maximum decrease taking place within first 24 hours. Thus, in the case of chapaties, stored at 8° C., there is a loss of 12.6% of reducing sugars in the first 24 hours, but this gradually increases later on and finally, after 72 hours, it returns nearly to the original value. Reducing sugars of chapaties stored at room temperature, on the other hand, do not show any such initial decrease after 24 hours. As a matter of fact, a progressive increase in the reducing sugar content starts taking place from the very beginning of the storage period.

There is a decrease in the water soluble polysaccharides of the chapaties in the first 24 hours in all the cases studied, irrespective of whether they are stored at high or low temperature or packaged in a plastic bag or in cloth. After this initial decrease, the soluble polysaccharides undergo very little change with time. It can be seen from the last column of the table that these calculations are also valid when the polysaccharide contents are expressed in terms of the constant soluble solids basis. This manner of expressing the data takes into account the simultaneous decreases in the polysaccharides and the total soluble solids contents of chapaties.

Results of analysis of chapaties wrapped in cloth in accordance with the local practice of preserving them for a day indicate that the pattern of the changes occurring therein is practically the same as that in chapaties packaged for 24 hours in an ordinary water-impervious heat sealable plastic pouch. There is a sudden drop in the contents of total solids and polysaccharides (measured as starch).

TABLE 1

Changes in water soluble solids and carbohydrates of chapaties stored under different conditions

Conditions of storage	Time of storage	Moisture	Grams per 100 g. of chapati					Starch on constant solid basis
			Total solids	Reducing sugars		Water soluble polysaccharides		
				Before hydrolysis	After hydrolysis	As dextrose	As starch	
Chapaties kept in sealed plastic bags and stored at room temperature	0 hours (fresh)	37.0	7.03	1.19	4.90	3.71	3.34	2.97
	24 hours	37.4	6.24	1.20	4.05	2.85	2.67	2.57
	48 hours	37.1	6.18	1.42	4.10	2.68	2.41	2.44
	72 hours	37.0	7.11	1.59	4.58	2.99	2.69	2.36
Chapaties kept in sealed plastic bags and stored in refrigerator (8° C.)	0 hours (fresh)	37.0	7.03	1.19	4.90	3.71	3.34	2.97
	24 hours	37.4	5.65	1.04	3.68	2.64	2.38	2.63
	48 hours	37.2	5.80	1.08	3.93	2.85	2.57	2.77
	72 hours	37.2	6.19	1.19	4.14	2.95	2.66	2.69
10 chapaties kept wrapped together in cloth at room temperature	0 hours (fresh)	39.5	7.12	1.05	5.02	3.97	3.57	3.13
	24 hours	36.5	6.33	1.12	4.29	3.17	2.85	2.81
Chapaties prepared according to the method developed at this Laboratory for long storage	0 hours	38.4	7.03	0.95	4.46	3.51	3.16	2.81
	72 hours	38.0	6.95	0.91	4.40	3.42	3.05	2.75
	About 6 months	33.2	6.76	1.72	3.65	1.93	1.74	1.61

Analysis of fresh and stored chapaties prepared and packaged according to the technique developed at this Laboratory indicates that there is a very pronounced reduction in the water-soluble polysaccharide content in chapaties stored for 6 months (Table 1). Reducing sugar content, on the other hand, greatly increases within this period. There was not much change in the total solids contents (Table 1).

Summing up all these observations, it can be said that irrespective of storage conditions and packaging methods, there is a decrease in the soluble polysaccharide content in chapaties during aging. The time within which this decrease is complete, however, varies—depending on whether any preservative has been used or not. In ordinary chapati, not supplemented with any preservative, the decrease is complete within the first 24 hours. Reducing sugar content of the chapaties stored under the various conditions also increases on aging. This increase is nearly 81% in the case of chapaties supplemented with preservative and stored for 6 months.

Thus, it appears that the nature of the changes taking place in the soluble carbohydrates of chapaties is virtually the same as that which has been reported in the case of bread during aging. A decrease in the soluble polysaccharides of bread, which is more or less complete within the first 17 hours, has been reported by Jackel *et al. (7)*, Bice and Geddes *(2)* and Schoch and French *(11)*. A progressive increase in reducing sugar has also been reported by Jackel *et al. (7)*. A progressive increase in water soluble solids in 72 hours of storage period has also been observed by Esselbaugh *(3)* and by Jackel *et al. (7)*.

The significance of these changes in chapaties (and also in bread) during aging is very little understood at the present time. A decrease in the soluble polysaccharides content can be attributed either to an enzymatic breakdown, to the reducing sugar or to an aggregation formed of large molecules which become insoluble. Increase in the reducing sugar content of chapaties, as observed by us, indicates that the first assumption may account partially for the changes in the polysaccharides. Investigations are also in progress at this laboratory to find out whether aging in chapaties is associated with changes in the molecular sizes of the polysaccharides present therein.

It has been alleged that the staling phenomenon in bread is closely associated with a decrease in the amount of soluble starch *(4, 10)*, though contrary opinions have also been expressed by other workers. Thus Jackel, Schaeder, and Schultz *(6)* on the basis of a decreased susceptibility of starch of bread to β-amylase as the criterion of staling, concluded that changes in soluble starch can not be related to staling phenomena. Our results indicate that, judged by the consumer tests, there is a correlation of the staling phenomenon with a decrease in soluble polysaccharides. Investigations to find out whether this decrease in soluble starch is in any way related to the change in the digestibility of starch of chapaties during staling are in progress in this laboratory.

2. Studies on the saccharides of chapaties and whole-wheat flour by circular paper chromatography. Studies on the changes in the polysaccharides and the reducing sugars of chapaties during storage, as reported in the previous section, reveal the net results of the changes undergone by

the various saccharides present therein, but they do not give any idea as to whether the changes occur in the different constituents equally or whether some particular component undergoes changes quite different in nature from those of the others. It was with this in mind that the chromatographic technique was employed to find an explanation of the changes taking place in sugars of chapaties stored for a very long time. We also took this opportunity to make a comparison of the saccharides of chapaties, fresh and stored, with those of whole wheat flour, fresh and stored.

The chromatogram of fresh chapaties indicates clearly the presence of sucrose, maltose, raffinose and a sugar giving a band intermediate in position between raffinose and maltose but having R_G slightly higher than that of melibiose (Fig. 1). This band is probably due to a gluco-fructosan as

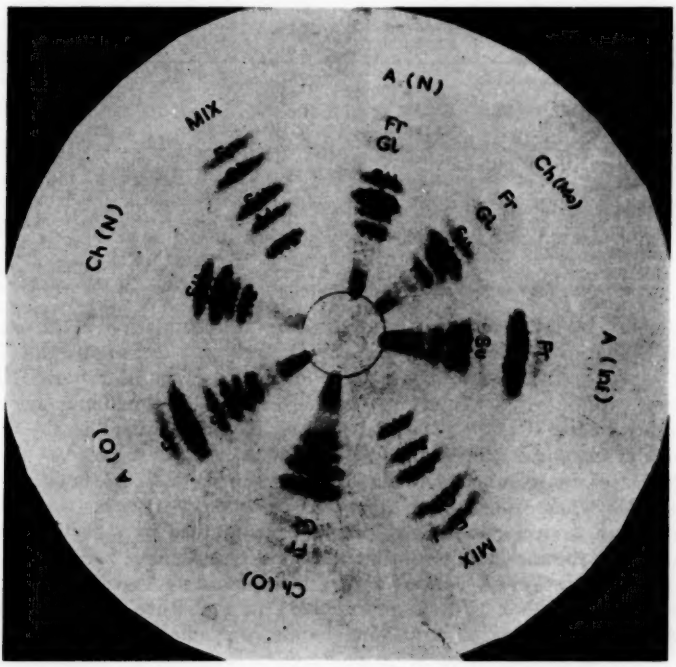

Figure 1. Saccharides of chapaties.

reported by Montgomery and Smith *(9)* and by Parihar *(10)*. Investigations are in progress to identify this compound. Further, on running the chromatogram for a very long time, it is possible to identify a number of bands due to higher saccharides having a D.P. (degree of polymerisation) of four, five and six glucose units. When compared to the chromatogram of whole wheat flour, the raw material from which chapati is made, one finds that in chapaties the band due to fructose is absent and that due to glucose is hardly visible. This points to a destruction of these or more

possibly to the formation of a sugar-protein complex during the baking operation. Other sugars which are present in chapaties are also present in wheat flour. The nature of the sugars in chapaties thus obtained is practically the same as that reported previously from this laboratory *(10)*. The R_G values of various sugars, as obtained in the present study are given in Table 2.

Chapaties stored for 6 months offered a picture almost identical to that of fresh chapaties, excepting that there is a reappearance of the bands due to glucose and fructose. Whether this is due to the degradation of the higher saccharides already present in chapaties or due to a breakdown of the glucose protein complex formed during baking, is yet to be ascertained.

TABLE 2

R_G values of various saccharides in chapaties

Saccharides	R_G
Glucose	1.00
Fructose	1.15
Sucrose	.77
Maltose	.633
Unknown band x	.55
Melibiose	.533
Raffinose	.466
D.P. 4	.266
D.P. 5	.216
D.P. 6	.166

Increase in the concentration of glucose and formation of fructose in stored chapaties as observed by the chromatographic technique, thus seems to be reproduced at least qualitatively in our findings as reported in the previous section with respect to the increase in the reducing sugars of chapaties during storage. The picture was almost identical in the case of samples of chapaties where some mold growth had taken place.

It was of interest to us to find out the corresponding changes in the saccharides when wheat flour was stored for a long time. As observed from Figure 1, storage of wheat flour is accompanied by a certain amount of change in the saccharide content, the most conspicuous being a decrease in the concentration of maltose with a corresponding increase in the concentration of glucose. Furthermore, there is a definite indication of the presence of a number of additional bands, presumably due to sugars of D.P. 4, 5, and 6, thereby showing an amylolysis of starch or other polysaccharides present in wheat flour during storage. This amylolysis effect is even more pronounced in the chromatogram of infested atta.

SUMMARY

Changes in water-soluble solids reducing sugars and polysaccharides of chapaties under different conditions of storage have been investigated.

Conditions of storage have much influence on those constituents. There is decrease in solids and polysaccharides of aqueous extract of chapaties after storage for 24 hours under different conditions, whereas reducing sugar in aqueous extract shows variable changes.

Storing chapaties for 6 months at room temperature results in a definite and pronounced increase in the reducing sugar content of the aqueous extract of chapaties while polysaccharides show an appreciable decrease.

Chromatographic analysis of sugars in water extract of chapaties stored for 6 months show that the increase in the reducing sugar is due to increase in the concentration of glucose and the formation of fructose.

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QUALITY IN BAKED SWEETPOTATOES AFFECTED BY VARIETIES AND POST-HARVEST TREATMENTS[a]

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As a standard procedure for minimizing storage losses sweetpotatoes are cured at about 85° F. (29° C.) in a relative humidity of 90 to 95% for at least 5 days before being stored at 55 to 60° F. (13 to 15° C.) to reduce the respiration rate. Even longer periods of 10 to 14 days curing are recommended in some publications *(3, 4)*. A large part of the commercial crop is harvested in late summer or early fall and sold for immediate consumption without curing. Uncured sweetpotatoes are desirable when boiled, canned, or made into pies, but are not generally accepted for baking until cured. Much has been written concerning the changes effected by curing and storing *raw* sweetpotatoes. Little can be found in the literature, however, on the effects of varieties, curing, and storage upon the quality of *cooked* sweetpotatoes, and this work, originally suggested by members of the Southern Cooperative Group, was undertaken to determine the effect of these factors.

METHODS

Sweetpotatoes for this study were grown in the Horticultural plots at State College, Mississippi, in 1954. The varieties Porto Rico and Allgold were harvested during the latter part of October, and roots were selected in the field for uniformity of size ranging between 2.5 and 3.0 inches in diameter and 4.0 to 5.0 inches in length. The selected roots were washed, dried, and pinned into a single layer within a commercial-type cabbage bag. These bags were suspended from wires near the ceiling in the curing room. A randomized order within 4 replications was followed in hanging the bags in the curing room. For 12 days following harvest the curing room temperature was held at about 85° F. with a relative humidity of 90% or higher. Then, the temperature was lowered to 55° F. with a relative humidity of about 70% which was maintained during the 14 weeks of storage.

Beginning immediately after harvest and at 2-day intervals throughout the 12-day curing period and at the end of 7 and 14 weeks storage samples of 10 roots from each variety within a replication were removed for carbohydrate analyses. At harvest, on the 6th and 12 days of curing, and after 7 and 14 weeks storage samples of 12 roots from each variety were removed from each replication, wrapped in aluminum foil, and baked in an oven at 325° F. for two hours. After baking, the roots were cooled for 30 minutes in crushed ice with the wrappers on. Ten of the baked roots were immediately sampled for carbohydrates and dry matter. The remaining two baked roots in each replication were stored at 0° F. in a home-type freezer for about 3 months until they were tested organoleptically. The roots were removed from the freezer, thawed, warmed, and split into halves. Halves of 3 roots composed a sample. The samples were paired and the test completed as described by Terry, Bradley, and Davis *(7)*. Two judges and two replications were employed.

In preparation for carbohydrate analysis 10 roots either raw or baked were split lengthwise in half. A half of each raw root was then split lengthwise with a mechanical

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rasp. Five grams of the rasped, macerated material was preserved in 80% ethanol to be analyzed at a later time. Ten grams of this material was taken for a dry matter sample. A V-shaped piece, closely approximating the cut made by the rasp in raw roots, was cut from a half of each baked root. The pieces were then macerated in a mortar, and samples were taken and treated as described for the raw roots. Sugars were determined by the method outlined by Pickett *(6)*. Analyses for starch were made as described by Loomis and Shull *(2)*. Dry matter was determined according to A.O.A.C. methods *(1)*. Storage weight loss was computed by weighing the individual roots at harvest and after curing and/or storage. All data were reported on a fresh weight basis, and each value is a mean of determinations from four samples.

RESULTS AND DISCUSSION

Total sugar content of raw Porto Rico sweetpotatoes was 5.6% as compared to 7.0% in Allgold according to the data shown in Table 1. Both reducing and non-reducing sugars increased during the first 6 days of the curing process. Longer curing periods and storage did not markedly affect

TABLE 1

Length of curing and storage affecting the sugars, starch, dry matter, and weight loss of raw Porto Rico and Allgold sweetpotatoes

Sampling times		Reducing sugars %	Non-reducing sugars %	Starch %	Dry matter %	Weight loss %
Cured days	Stored weeks					
Porto Rico						
Harvest		0.03	2.96	10.91	28.2	
2	0	0.03	3.73	11.74	27.0	2.2
4	0	0.06	4.77	12.31	29.7	6.7
6	0	0.16	6.33	11.65	32.0	9.0
8	0	0.22	6.51	11.41	30.5	9.8
10	0	0.16	6.00	11.25	31.0	10.4
12	0	0.14	5.63	11.80	31.1	12.9
12	7	0.23	6.12	9.64	29.9	14.5
12	14	0.41	6.89	5.29	29.5	14.7
LSD @ 5%		0.7	0.72	1.79	NS	1.5
Average		0.16	5.43	10.66	29.9	10.0
Allgold						
Harvest		0.08	4.76	7.43	24.6	
2	0	0.02	5.47	8.63	25.1	2.1
4	0	0.08	6.59	7.81	25.8	5.8
6	0	0.09	7.60	7.99	26.6	9.9
8	0	0.12	7.25	7.36	26.2	8.3
10	0	0.14	7.04	8.01	26.9	9.5
12	0	0.11	7.60	8.42	26.3	9.5
12	7	0.15	7.26	5.85	25.1	15.7
12	14	0.12	8.13	8.14	24.3	16.0
LSD @ 5%		0.07	0.59	1.13	1.60	0.2
Average		0.10	6.86	7.74	25.7	9.6
LSD @ 5% between varietal means		0.04	0.12	0.28	1.69	NS

the sugar content of the raw roots. Starch was more abundant in the raw Porto Rico than in Allgold. After 7 weeks' storage, starch decreased in Porto Rico but remained unchanged in Allgold throughout the 14 weeks of storage.

More dry matter was present in Porto Rico than in Allgold roots. The dry matter content changed little by curing and storing the sweetpotatoes. When the raw roots were baked there was a slight increase in dry matter content.

Each variety lost about the same amount of weight when cured and stored. The longer the storage period, the greater the weight loss. This loss was likely caused by the decrease of carbohydrates through respiration and by storage-rot organisms.

Baked Porto Rico roots contained 10.1% sugars and Allgold 9.2% (Table 2). Baking caused the reducing sugars to be increased from less

TABLE 2

The effect of curing and storage upon sugars, starch, and dry matter contained in baked Porto Rico and Allgold sweetpotatoes

Sampling times		Reducing sugars %	Non-reducing sugars %	Starch %	Dry matter %
Cured days	Stored weeks				
		Porto Rico			
Harvest	0	1.92	0.76	5.68	31.6
6	0	2.00	8.14	5.21	34.6
12	0	1.92	7.85	5.58	34.8
12	7	3.84	6.99	3.96	32.7
LSD @ 5%		0.56	3.75	0.74	NS
Average		2.42	7.65	5.11	33.4
		Allgold			
Harvest	0	1.08	4.08	4.76	28.4
6	0	1.24	10.88	3.71	29.6
12	0	1.68	6.99	4.21	30.6
12	7	2.68	8.29	2.07	26.9
LSD @ 5%		0.52	1.85	0.46	2.5
Average		1.67	7.56	3.69	28.9
LSD @ 5% between varietal means		0.12	NS	0.20	1.5

than 0.1% in the raw to 2 or 3% in the baked roots; however, this increase did not occur in the non-reducing sugar fraction. Curing the roots for as much as 6 days caused the roots to have four times as much reducing sugar as the uncured, baked roots. Allgold roots cured for 6 days before baking had only a two-fold increase in reducing sugars. For either variety curing longer than 6 days and storage for 14 weeks did not radically change the sugar content of the roots when baked.

About half of the starch in the raw roots was converted to sugar in baking. This starch conversion to sugars was even more than half in the

TABLE 3

Percentage of times baked sweetpotatoes would be first choice organoleptically for various attributes

Sampling times		Most orange color %	Most uniform color %	Best holding of shape %	Most sweet %	Most fine texture %	Most moist %
Cured days	Stored weeks						
				Porto Rico			
Harvest		25	35	78	2	7	5
6	0	16	6	6	18	26	27
12	0	40	48	11	11	16	17
12	7	3	7	3	18	10	17
12	14	16	4	2	51	41	34
Probability percentage		1.8	0.5	0.01	0.8	9.2	9.8
				Allgold			
Harvest		33	28	78	4	4	1
6	0	11	13	15	22	16	18
12	0	33	19	3	39	22	33
12	7	14	16		30	50	46
12	14	9	24	4	5	8	2
Probability percentage		19	79		0.5	0.9	

roots stored for 7 weeks, indicating an increase with time in the effectiveness of the responsible enzyme.

Organoleptic values (Table 3) indicate roots of either variety baked at harvest or after 12 days curing had the most orange color. Roots of both varieties held their shapes better when baked immediately after harvest than when cured or stored before baking. Porto Rico baked after 14 weeks' storage and Allgold after 7 weeks' storage had the finest texture and moistest taste of all the other baked lots.

The primary reasons for curing sweetpotatoes before storage are to form wound periderm in the prevention of storage losses and to increase the sugar content for a better tasting product when baked. A 5-day curing period was found by Moore and Anderson *(5)* to be sufficient before the roots were stored in contrast to 10 to 14 days as commonly used and recommended. Six days curing as shown here is sufficient for the conversion of starch to sugar and to improve flavor and culinary qualities. By using a shorter curing period the grower can save fuel and personal attention that was formerly thought necessary to properly cure sweetpotatoes.

Flavor is likely affected by the principal sugars present in the baked roots. The amount and kind of sugars formed from starch as a sweetpotato is baked appear to vary with the variety. Differences percentage-wise in the two sugar fractions of the varieties in this study do not seem very great. Yet, slight differences in flavor will affect consumer preferences and may in turn affect drastically the economic values of sweetpotato varieties. The familiar flavor may explain why Porto Rico is the leading variety grown in the southern states and why it is so difficult to replace it with a newer, better yielding, more attractive variety. Further work is needed

to fully understand what specific sugars are included in the reducing and non-reducing fractions of various sweetpotato varieties and how these sugars influence consumer preferences.

SUMMARY

The raw sweetpotatoes of both varieties increased in reducing and non-reducing sugars during the first six days of the curing period but changed little after that time in curing and storage. Baking increased the reducing sugar content from about 0.1% to 2 or 3% in the roots of each variety. Only about half as much starch was contained in the roots after baking as compared to the original. Dry matter changed little with storage or baking. Weight losses in storage for each variety were about equal.

Organoleptic tests revealed that baked Allgold roots were more uniformly colored orange than Porto Rico. Allgold cured and stored for 7 weeks and cured Porto Rico stored for 14 weeks had a finer texture and were more moist than any of the cured and/or stored lots when baked. Quality in the baked roots was dependent upon the variety and length of curing and storage times employed.

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I. EFFECT OF STORAGE AND OF BOILING ON THE ASCORBIC, DEHYDROASCORBIC, AND DIKETOGULONIC ACID CONTENTS OF POTATOES[a]

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Numerous dietary surveys have shown that potatoes are an important source of ascorbic acid for low-income groups. That storage results in serious losses of this nutrient also is well established from work in several laboratories *(4)*. Furthermore, various methods of home cooking have been shown to result in an appreciable reduction in the ascorbic acid content of the prepared products *(2, 3, 4)*. However, relatively little information is available as to the extent to which these storage and cooking losses can be accounted for by the conversion of reduced ascorbic acid to dehydroascorbic and to diketogulonic acids. Evidence that dehydroascorbic acid is an effective source of vitamin C for human subjects *(1, 11)*, whereas diketogulonic acid is not, suggested the importance of investigating the relative quantities of these three compounds in potatoes under various conditions of storage and following several methods of cooking. The results with boiled potatoes are reported in this paper.

PROCEDURE

For the study on boiled potatoes two varieties, Chippewa and Triumph, were used. The tubers were purchased on the retail market in November and December. As soon as they arrived in the laboratory they were sampled for immediate analysis. The remainder was treated as follows: 6 tubers of approximately equal size were stored at 24° C. (room temperature) for 3 weeks and then analyzed, the balance was held in cold storage at 1° C. and at the end of 3 weeks, two lots of 6 tubers each were removed. One lot was analyzed immediately, and the other was held at room temperature for 3 weeks before analysis. Additional lots were removed from cold storage at 3-week intervals and handled in the same manner. Each variety was studied for 15 weeks.

Tubers stored continuously at 1° C. were removed from storage 24 hours before analysis and held at room temperature during this time. On the afternoon prior to analysis the potatoes were washed thoroughly, rinsed with distilled water, and blotted dry with a towel. The following morning the tubers were peeled and sampled immediately. Thin wedges were cut longitudinally from the bud to the stem end. Each composite sample, of which there were four, included sectors from each of the six potatoes. Two of these samples were used for ascorbic acid analysis and two for moisture determinations. After the pared potatoes had been sampled for analysis in the raw state, one quarter from each of the 6 tubers was cooked on an electric hot plate in a covered aluminum pan in 200 ml. of unsalted, distilled water until done as tested by the tines of a fork. During the cooking process the cover of the pan was tilted slightly to permit escape of steam. Average length of cooking time was 20 minutes.

After the tubers were removed from the heating unit they were cooled as rapidly as possible by placing the pan in cold water. They were then mashed quickly with a stainless steel masher; any water remaining in the pan was incorporated into the

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mashed potatoes. Preliminary tests had shown that with this amount of water there would be only a few milliliters remaining when the potatoes were "done." This eliminated losses in ascorbic acid due to solution in the cooking water.

To study the effect of holding the cooked potatoes, aliquots of the cooled, mashed tubers were placed immediately in a refrigerator in a covered glass dish and analyzed 24 hours later.

Duplicate aliquots of the cooked and the raw tubers, 15 and 25 g., respectively, were triturated with acid-washed sand and a cold 10% solution of stannous chloride in 3% metaphosphoric acid. After extraction, enough metaphosphoric acid solution was added to make a 0.5% stannous chloride solution and the extract was filtered. The contents of ascorbic, dehydroascorbic, and diketogulonic acids of the filtrate were determined according to the methods of Roe, Mills, Oesterling, and Damron *(10)*.

Moisture contents of the raw and cooked potatoes were estimated by drying 100 g. aliquots to constant weight at 90° C. in steam-heated drying drawers. All results are expressed on a dry weight basis to permit direct comparison between the values for the raw and cooked potatoes. Analysis of variance was used to test the significance of the observed differences.

RESULTS AND DISCUSSION

Effect of storage, storage temperature, and boiling. *Ascorbic Acid.* Tables 1 and 2 show that the Chippewa and Triumph potatoes which were held at 1° C. retained slightly more than one-half—59% and 55%, respectively—of their original ascorbic acid content at the end of 15 weeks of storage. Losses occurred continuously throughout the storage period and were the most rapid during the first 6 weeks. These losses were found to be significant at the 1% level. For the potatoes held for 3 weeks at 24° C. following storage at 1° C., losses attained the 5% level of significance. In terms of retention it was found that the Chippewa variety retained 65% of its original ascorbic acid value at the end of the study and Triumph, 67%. Except for the values for Chippewa variety after 6 weeks of storage, tubers which were held for 3 weeks at 24° C. following storage at 1° C. had higher ascorbic acid values than did those held continuously at the low temperature. Differences attributable to storage temperature were significant at the 5% level. This is in agreement with our earlier observation *(4)* that tubers which are held for a period at a higher temperature appear to recover a part of the ascorbic acid which is lost during storage at low temperatures.

Highly significant increases in ascorbic acid were noted in both varieties of potatoes as a result of boiling. These increments were surprisingly high and were considerably in excess of those reported by some workers *(4, 9, 12)*, but not as great as those observed by others *(7)*. A number of possible explanations have been advanced for these reported increases *(3, 5, 8)*. In the present study the most feasible are: (1) the ascorbic acid is more easily extracted from the softened cooked vegetable than from the raw tissues, and (2) "bound" ascorbic acid present in the raw tubers is not extracted by customary procedures but is hydrolyzed during the cooking process.

Since the potato tissue had been triturated with sand to avoid conversion of ascorbic acid to the dehydro form, as suggested by Roe, Mills, Oesterling, and Damron *(10)*, it seemed possible that extraction of the raw tissue was less complete than if the tissue had been comminuted in a blender. To test this hypothesis, a series of determinations was carried out

TABLE 1

Moisture, ascorbic acid, dehydroascorbic acid, and diketogulonic acid values for Chippewa variety potatoes—analyzed raw, immediately after cooking, and after 24 hours of refrigerator storage of the cooked potatoes

	Stored at 1° C.					
	0 wks	3 wks	6 wks	9 wks	12 wks	15 wks
Moisture (%)						
Raw	79.9	80.4	79.4	79.3	81.8	78.7
Cooked	81.2	79.8	81.4	79.6	80.5	79.4
Ascorbic acid (mg./100 g. dry wt.)						
Raw	44.0	32.5	31.8	24.3	24.8	25.9
Cooked, analyzed immediately	90.0	70.8	51.1	38.9	44.6	32.7
analyzed after 24 hr. refrig.	41.1	48.1	37.4	37.0	31.7	26.6
Dehydroascorbic acid (mg./100 g. dry wt.)						
Raw	10.0	5.5	4.0	5.0	5.8	1.1
Cooked, analyzed immediately	1.1	5.8	0.0	0.0	6.5	0.0
analyzed after 24 hr. refrig.	6.5	10.2	13.4	1.8	6.5	0.0
Diketogulonic acid (mg./100 g. dry wt.)						
Raw	4.6	4.4	2.7	1.1	1.0	2.5
Cooked, analyzed immediately	10.6	4.4	7.4	15.8	0.4	12.8
analyzed after 24 hr. refrig.	9.0	13.9	9.5	13.1	6.7	17.1

	Stored at 1° C. followed by 3 wks. at 24° C.					
	0 wks	3 wks[1]	3 wks	6 wks	9 wks	12 wks
Moisture (%)						
Raw	79.9	78.8	78.1	79.5	79.1	78.3
Cooked	81.2	78.9	81.0	79.8	79.4	79.5
Ascorbic acid (mg./100 g. dry wt.)						
Raw	44.0	39.8	30.6	30.7	31.4	28.4
Cooked, analyzed immediately	90.0	67.2	66.4	44.2	54.1	47.7
analyzed after 24 hr. refrig.	41.1	48.5	47.5	24.6	43.9	37.0
Dehydroascorbic acid (mg./100 g. dry wt.)						
Raw	10.0	6.2	6.3	5.6	4.6	4.3
Cooked, analyzed immediately	1.1	4.6	2.9	0.0	0.0	0.0
analyzed after 24 hr. refrig.	6.5	16.9	9.6	8.7	6.9	5.2
Diketogulonic acid (mg./100 g. dry wt.)						
Raw	4.6	3.5	1.7	1.4	1.3	1.2
Cooked, analyzed immediately	10.6	6.7	4.7	11.1	5.0	11.4
analyzed after 24 hr. refrig.	9.0	9.7	9.2	14.1	8.3	12.2

[1] Stored only at 24° C. for 3 weeks.

in which these two methods of extraction were compared. The results showed that there was no significant difference in the reduced ascorbic acid values obtained by the two methods. However, approximately 5% more total ascorbic acid (reduced plus dehydroascorbic acid) was extracted with the blender. Essentially all of this increase was in the dehydro form. It is possible that longer comminution in the blender than was used in this study (2 minutes) would have yielded a still higher return, but since the indications were that this also would have resulted in a conversion of additional amounts of ascorbic acid to the dehydro form, no further tests were carried out.

Although Hewston, Dawson, Alexander, and Orent-Keiles *(2)* reported negative results in tests for protein-bound ascorbic acid in potato tubers,

TABLE 2

Moisture, ascorbic acid, dehydroascorbic acid, and diketogulonic acid values for Triumph variety potatoes—analyzed raw, immediately after cooking, and after 24 hours of refrigerator storage of the cooked potatoes

	Stored at 1° C.					
	0 wks	3 wks	6 wks	9 wks	12 wks	15 wks
Moisture (%)						
Raw	81.2	79.0	81.2	81.2	80.2	80.3
Cooked	81.8	80.4	81.6	79.6	79.2	81.6
Ascorbic acid (mg./100 g. dry wt.)						
Raw	42.9	42.2	33.9	30.8	27.4	23.7
Cooked, analyzed immediately	82.1	75.8	56.7	34.1	35.0	39.0
analyzed after 24 hr. refrig.	54.0	62.2	37.7	22.6	28.2	23.2
Dehydroascorbic acid (mg./100 g. dry wt.)						
Raw	8.1	9.7	5.3	6.6	5.4	4.8
Cooked, analyzed immediately	0.3	0.0	0.0	0.0	0.0	0.0
analyzed after 24 hr. refrig.	11.2	18.6	8.7	13.5	0.0	13.4
Diketogulonic acid (mg./100 g. dry wt.)						
Raw	3.8	1.8	4.4	4.6	4.6	2.3
Cooked, analyzed immediately	8.7	15.9	8.8	12.4	9.6	12.5
analyzed after 24 hr. refrig.	16.6	10.4	12.0	11.0	16.2	11.7

	Stored at 1° C. followed by 3 wks. at 24° C.					
	0 wks	3 wks[1]	3 wks	6 wks	9 wks	12 wks
Moisture (%)						
Raw	81.2	78.5	79.6	79.7	81.3	77.9
Cooked	81.8	80.2	80.0	81.2	83.6	80.6
Ascorbic acid (mg./100 g. dry wt.)						
Raw	42.9	36.8	36.4	35.9	38.6	28.8
Cooked, analyzed immediately	82.1	72.2	67.6	57.5	59.5	55.5
analyzed after 24 hr. refrig.	54.0	62.8	51.9	49.0	51.2	48.0
Dehydroascorbic acid (mg./100 g. dry wt.)						
Raw	8.1	5.4	6.8	4.1	2.9	4.9
Cooked, analyzed immediately	0.3	0.0	2.0	0.1	0.0	0.9
analyzed after 24 hr. refrig.	11.2	8.7	9.9	4.7	5.9	9.4
Diketogulonic acid (mg./100 g. dry wt.)						
Raw	3.8	2.8	1.4	2.3	3.7	2.4
Cooked, analyzed immediately	8.7	14.8	4.6	5.7	11.3	9.0
analyzed after 24 hr. refrig.	16.6	11.3	7.0	12.5	13.3	8.1

[1] Stored only at 24° C. for 3 weeks.

it is possible that length of time since harvest, conditions of storage, and other factors might account for the fact that apparently substantial amounts of "bound" ascorbic acid occurred in the tubers used in the present study.

Dehydroascorbic Acid. Both varieties of potatoes contained appreciable amounts of dehydroascorbic acid at the time they were brought into the laboratory. For the Chippewa variety the dehydro form constituted 18% of the total ascorbic acid in the raw tissue and for the Triumph variety, 16%. Throughout storage, decreases in the amount of dehydroascorbic acid occurred with fair regularity in the two varieties and at both storage temperatures.

Except in two instances, boiling reduced markedly the dehydroascorbic acid content of the tubers. Generally the boiled potatoes contained negligible amounts or none of the oxidized form. This observation is in accord with the findings of Noble and Hanig *(6)* who reported that, in a variety of vegetables tested by several cooking methods, there was always less dehydroascorbic acid in the cooked than in the raw vegetable.

Diketogulonic Acid. Diketogulonic acid determinations were made to ascertain the extent to which the losses in ascorbic and dehydroascorbic acids which occurred during storage and as a result of cooking could be accounted for by increases in diketogulonic acid. Tables 1 and 2 show that both varieties of potatoes contained appreciable amounts of diketogulonic acid at the time they were received in the laboratory. For the Chippewa variety during the first two storage periods, there was a rapid reduction in this factor in the raw tissue with less pronounced changes thereafter. For the Triumph variety, changes in diketogulonic acid content showed no relationship to storage time, nor could they be correlated with changes in the other two factors studied. It is evident that the losses in ascorbic and dehydroascorbic acid during storage are not accounted for by corresponding increases in diketogulonic acid.

The diketogulonic acid content of both varieties of tubers increased markedly when the potatoes were boiled. When tested statistically, these increases were found to be highly significant. Because of the observed increases in ascorbic acid in the boiled potatoes, it was not possible to ascertain the source or sources of the increment in diketogulonic acid in these tubers.

Effect of 24-hours' refrigeration on boiled potatoes. *Ascorbic Acid.* Cooked potatoes of both varieties which were held in a refrigerator for 24 hours showed a marked loss in ascorbic acid compared with the freshly cooked tubers. When tested statistically the differences were found to be highly sigificant. Although for the Triumph the losses in ascorbic acid due to refrigeration appear to be greater for the tubers stored at 1° C. than for those held for 3 weeks at 24° C. following storage at the lower temperature, differences were not statistically significant.

Chippewa which had been stored at 24° C. lost a significantly greater amount of ascorbic acid on refrigeration of the cooked product than did Triumph variety. This was not true of the tubers stored at 1° C.

Dehydroascorbic Acid. Refrigeration of cooked potatoes usually resulted in marked increases in dehydroascorbic acid compared with the freshly cooked product. The values obtained on the two varieties of refrigerated tubers during the 15 weeks of study indicated that this component constituted on the average about one-eighth of the total ascorbic acid content of the tubers; however, in some instances dehydroascorbic acid made up as much as one-third of the total.

The fact that a considerable portion of the ascorbic acid lost on refrigeration was present in the dehydro form makes potatoes which are held in a refrigerator for no more than 24 hours an appreciably better antiscorbutic than would appear to be the case when only reduced ascorbic acid values are considered.

Diketogulonic Acid. Cooked potatoes which were refrigerated for 24 hours generally showed increases in diketogulonic acid. However, owing to the variability in results these changes were not statistically significant.

SUMMARY

Ascorbic, dehydroascorbic, and diketogulonic acid values for two varieties of potato tubers, Chippewa and Triumph, purchased from a local market, were determined on raw and boiled potatoes, and on boiled potatoes which had been refrigerated for 24 hours. Analyses were made at three-week intervals for 15 weeks on tubers which were stored continuously at 1° C., and on tubers which had been held for three weeks at 24° C. following storage at 1° C.

Storage for 15 weeks resulted in a significant reduction in the ascorbic acid content of the raw potato tissue. Potatoes stored continuously at 1° C. contained significantly less ascorbic acid than did those held for the last three weeks of storage at 24° C.

Ascorbic acid values for the boiled potatoes were significantly higher than those for the corresponding raw tubers. The implications of this observation have been discussed. Cooked potatoes which were stored in a refrigerator for 24 hours contained significantly less ascorbic acid than the freshly cooked product.

At both storage temperatures, the dehydroascorbic acid value for raw potato tissue decreased with time. Boiling resulted in an almost complete loss of dehydroascorbic acid. Refrigeration of boiled potatoes, on the other hand, increased markedly the dehydroascorbic acid content as compared with that of the freshly cooked tubers.

The diketogulonic acid content of raw potato tissue also decreased with storage, from which it is evident that the storage losses in ascorbic and dehydroascorbic acids are not offset by equivalent increases in diketogulonic acid. Boiling, on the other hand, resulted in a significant increase in diketogulonic acid, with a further increase in most cases as a result of 24-hours' refrigeration of the boiled tubers.

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II. EFFECT OF BAKING AND OF PRESSURE-COOKING ON THE ASCORBIC, DEHYDROASCORBIC, AND DIKETOGULONIC ACID CONTENTS OF POTATOES[a]

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Several reports have appeared in the literature, which indicate that significant losses in ascorbic acid result from the home cooking of potatoes. In most of these studies, the products were assayed for reduced ascorbic acid only. In some of the investigations in which total ascorbic acid (reduced plus dehydroascorbic acid) was determined, a considerable amount of the dehydroascorbic acid was found in the cooked product *(1, 2, 3, 6)*. Since the latter has been shown to be physiologically available for the human adult *(5)* it would seem essential in any vitamin C assay to determine the dehydro as well as the reduced ascorbic acid content. In the present study the effect of baking and of cooking in a pressure saucepan on the ascorbic and dehydroascorbic acid contents of potatoes was determined. In order to ascertain the extent to which losses in total ascorbic acid can be ascribed to increases in diketogulonic acid, the latter component also was estimated.

PROCEDURE

For the baking tests Red Warba, Washington Russet, and Montana-grown Triumph potatoes were secured from a local market in November and stored at 4.5° C. For each baking test three lots of 6 tubers each were used. The potatoes were washed thoroughly, rinsed with distilled water, and blotted dry with paper towels. Lot 1 was analyzed raw; lots 2 and 3 were baked in an electric roaster to an internal temperature of 99.5° C. (about 45 minutes). Lot 2 was analyzed immediately; lot 3 was allowed to cool spontaneously to room temperature (about 2 hours), stored in a refrigerator overnight, and analyzed the following day. The experiment was repeated four times on each variety.

Raw potatoes were sampled by paring and cutting a thin wedge from the bud to the stem end from each of the 6 tubers. Duplicate 25 g. composite samples were transferred to blenders, covered immediately with 10 ml. of 10% stannous chloride, followed by 190 ml. of cold 5% metaphosphoric acid, and triturated for 2 minutes.

The baked potatoes which were analyzed immediately (lot 2) were split lengthwise, the contents scooped out of the skins into a 1-quart aluminum saucepan, cooled rapidly by placing the pan in cold water, and mashed quickly using a stainless steel masher. Two samples of 15 g. each were weighed and extracted in the same manner as the raw potatoes. The baked potatoes which had been stored in the refrigerator overnight were handled similarly. Great care was taken to avoid including any browned tissue in the samples.

For the study on potatoes cooked in a pressure saucepan, tubers of the Kennebec variety, Minnesota-grown, were purchased in mid-November from a local market, and those of the Redcote variety, from a commercial grower located in the Red River Valley. The latter were delivered to the laboratory the last week of November. Both varieties were stored at room temperature throughout the experiment. The tubers were handled as follows: two lots of four tubers each were prepared prior to sampling in

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the same manner as the tubers used in the baking experiment. One lot was analyzed raw, the other lot was used for the cooking test. The latter were peeled, halved, placed on a rack in a 3-quart aluminum pressure saucepan, and cooked at 15 pounds pressure for 8 minutes. In addition to the 8 minutes' cooking time, there was an additional period of 4 minutes which was necessary for bringing to a boil the ½ cup of distilled water added to the cooker, and for allowing the air and steam to escape (1 minute) before the indicator-weight was attached. As soon as cooking was completed, the pressure saucepan was cooled immediately under cold water; this caused the pressure to drop to zero within 30 to 45 seconds. The saucepan was opened and one of the halves from each potato was removed and placed in a 1-quart aluminum saucepan. This pan was placed in cold water and the tubers were cooled quickly and mashed with a stainless steel masher. Samples for analysis were weighed at once. The other 4 halves were left in the pressure saucepan and allowed to cool to room temperature spontaneously. These were mashed and aliquots were weighed, placed in a glass refrigerator dish, covered, and refrigerated for 24 hours. Similar aliquots of the tubers which were cooled quickly were prepared and refrigerated. Analyses were made the following day. The tests on the pressure-cooked potatoes were repeated 4 times. On the Kennebec variety the study was completed within 14 days of the purchase of the tubers, on the Redcote variety, within 22 days.

Duplicate determinations were made on each sample of the ascorbic acid, dehydroascorbic acid and diketogulonic acid contents using the procedure of Roe, Mills, Oesterling, and Damron *(4)*. The moisture contents of the raw and cooked potatoes were determined by air-drying 100 g. aliquots to constant weight in steam-heated drying drawers. Analysis of variance was used to test the significance of the observed differences.

RESULTS AND DISCUSSION

Effect of baking and of refrigeration after baking. The effects of baking and of holding in a refrigerator for 24 hours after baking on the contents of ascorbic, dehydroascorbic, and diketogulonic acids of 3 varieties of potatoes are shown in Table 1. The data are reported on the dry weight

TABLE 1

Moisture, ascorbic acid, dehydroascorbic acid, and diketogulonic acid values for three varieties of potatoes: raw, baked, and refrigerated[1]

	Red Warba	Washington Russet	Triumph	Mean
Moisture (%)				
Raw	81.7	75.4	75.5	77.5
Baked	81.1	74.4	74.3	76.6
Refrig.	82.3	75.5	75.1	77.6
Ascorbic acid (mg./100 g. dry wt.)				
Raw	36.5	49.7	54.5	46.9
Baked, cooled immediately	48.0	56.5	48.5	51.0
cooled slowly, refrig. 24 hrs.	18.8	28.4	12.0	19.8
Dehydroascorbic acid (mg./100 g. dry wt.)				
Raw	17.5[2]	13.8	13.3	14.6
Baked, cooled immediately	6.4[2]	1.0	5.0	4.1
cooled slowly, refrig. 24 hrs.	19.2	8.9	16.3	14.8
Diketogulonic acid (mg./100 g. dry wt.)				
Raw	4.8[2]	3.0	1.4	2.9
Baked, cooled immediately	10.1	8.0	9.0	8.9
cooled slowly, refrig. 24 hrs.	20.4	24.2	21.8	22.2

[1] Each value is the mean of four observations, except as otherwise noted.
[2] Mean of three observations.

basis since small changes in the amount of moisture occurred with baking and refrigeration.

Ascorbic acid. A comparison of the ascorbic acid values of the baked potatoes with the values for the raw tubers shows that for two varieties, Red Warba and Washington Russet, increases occurred on baking, whereas for Triumph variety, baking resulted in a decrease in this component. When tested statistically, the mean increase was not significant because the varieties did not respond to baking in the same manner.

Of the 3 varieties chosen for the baking test, Washington Russet variety is customarily sold as a baking potato, Triumph as a general purpose potato, and Red Warba as a boiling potato. From Table 1 it may be noted that Washington Russet and Triumph were very similar in moisture content, whereas Red Warba was considerably higher. If percentage of dry matter were a factor in ascorbic acid retention, it would be expected that the Washington Russet and Triumph potatoes would have responded similarly to baking. However, as was pointed out earlier, this was not the case.

All 3 varieties showed highly significant losses in ascorbic acid as a result of 24-hours' refrigeration of the cooked tubers. The mean retention for the varieties studied was 39% of the amount present in the freshly baked product.

Dehydroascorbic acid. Substantial amounts of dehydroascorbic acid were found in the raw tubers of all 3 varieties. Although every precaution was taken in the preparation and extraction of the samples, it is possible that these high values for dehydroascorbic acid may be attributable, at least in part, to the use of a blender for comminution of the samples as was suggested by Roe and coworkers. It should be pointed out, however, that in an earlier study potatoes which were extracted by trituration with sand also contained appreciable, although somewhat smaller, amounts of dehydroascorbic acid.

All 3 varieties of potatoes showed marked decreases in dehydroascorbic acid after baking. Statistically, these losses were highly significant. The mean retention after baking was 34% of the amount present in the raw tuber.

In every instance 24-hours' refrigeration of the baked potato induced highly significant increases in the dehydroascorbic acid content of the tubers. The mean value for the 3 varieties represented more than a threefold increase over the amount found in the freshly baked product.

Diketogulonic acid. Small amounts of diketogulonic acid were found in the raw samples of all 3 varieties studied. Baking the potatoes resulted in highly significant increases in this component. Triumph tubers, the only variety which lost ascorbic acid when baked, showed the greatest increase in diketogulonic acid. The mean diketogulonic acid value for the 3 varieties was three times that found in the raw tubers.

Refrigeration of the baked potatoes induced further marked increments in diketogulonic acid. These increases were statistically significant at the 1% level.

Effect of pressure cooking and of refrigeration after cooking. The ascorbic acid, dehydroascorbic acid, and diketogulonic acid values for Kennebec and Redcote potato tubers which were cooked in a pressure saucepan are shown in Table 2.

TABLE 2

Moisture, ascorbic acid, dehydroascorbic acid, and diketogulonic acid values for two varieties of potatoes: raw, cooked in a pressure saucepan and refrigerated after two methods of cooling[1]

	Kennebec	Redcote	Mean
Moisture (%)			
Raw	78.7	77.4	78.0
Cooked, cooled immediately	77.0	75.7	76.4
cooled slowly	76.0	75.7	75.8
Ascorbic acid (mg./100 g. dry wt.)			
Raw	75.4	48.6	62.0
Cooked, cooled immediately	70.4	47.2	58.8
cooled immediately, refrig. 24 hrs.	36.6	16.1	26.3
cooled slowly, refrig. 24 hrs.	27.0	15.4	21.2
Dehydroascorbic acid (mg./100 g. dry wt.)			
Raw	20.2	16.1	18.2
Cooked, cooled immediately	5.7	2.7[2]	4.4
cooled immediately, refrig. 24 hrs.	26.0	20.5	23.2
cooled slowly, refrig. 24 hrs.	20.4	9.2	14.8
Diketogulonic acid (mg./100 g. dry wt.)			
Raw	1.8	2.4	2.1
Cooked, cooled immediately	8.3	8.5[2]	8.4
cooled immediately, refrig. 24 hrs.	18.2	18.5	18.3
cooled slowly, refrig. 24 hrs.	23.8	25.9	24.8

[1] Each value is the mean of four observations, except as otherwise noted.
[2] Mean of three observations.

Ascorbic acid. For both varieties of potatoes, retention of ascorbic acid was high in the pressure-cooked tubers; however, severe losses in this component resulted from 24-hours' refrigeration of the pressure-cooked tubers. The mean retention for the tubers which were cooled quickly was 45% and for those which were allowed to cool spontaneously, 36% of the amount present in the freshly cooked product. These changes in the ascorbic acid content of the tubers as a result of refrigeration were highly significant.

Dehydroascorbic acid. On the average 22% of the total ascorbic acid in the raw tubers of the two varieties was in the dehydro form. Cooking the potatoes in a pressure saucepan caused highly significant losses of this component; the mean retention was only 24% of the amount present in the raw tubers.

Refrigeration of the tubers, which were cooled as rapidly as possible, resulted in a highly significant increase in the dehydroascorbic acid content of both varieties compared with the amount in those assayed immediately after cooking. Tubers cooled slowly before refrigeration contained less dehydroascorbic acid than did those cooled rapidly. For the Kennebec variety this difference in the response to the two methods of cooling before refrigeration was not significant; for the Redcote tubers, the difference was highly significant.

Diketogulonic acid. Raw potatoes of both varieties contained small amounts of diketogulonic acid. Cooking in a pressure saucepan resulted in significant increases in this component with further increases on refrigeration. These changes were more pronounced in those tubers allowed to

cool spontaneously than in those cooled quickly. In both instances, however, the increases were highly significant.

Distribution of the three components. The extent of the conversion of ascorbic acid to dehydroascorbic acid and to diketogulonic acid cannot be stated in definite amounts since the sum of the 3 components decreased as the potatoes were cooked and refrigerated. It is clear that with each treatment there was a conversion of some of the compound previously capable of coupling with 2, 4-dinitrophenylhydrazine into a substance which is not measured by this method. Figure 1 gives the sum and the distribution in

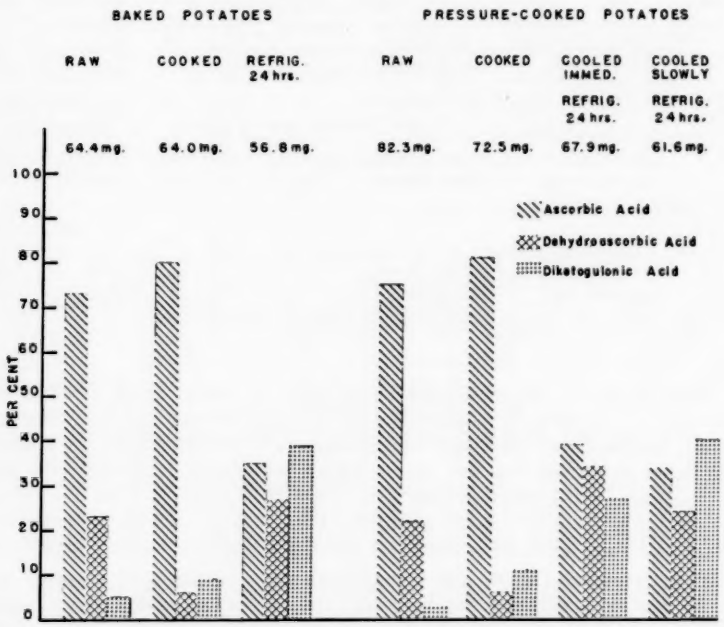

Figure 1. Distribution of ascorbic, dehydroascorbic, and diketogulonic acids in potato tubers treated in various ways, expressed as percentages of the sums of the three components. These sums are given under the method of treatment and are the means for two or more varieties of potatoes.

percentage of the individual components. These percentages were calculated from the means for the varieties studied for each treatment. It is evident that when the potatoes were cooked the primary change was the loss of dehydroascorbic acid. In the refrigerated potatoes which were cooled quickly after cooking, an increased percentage of the total coupling compound appeared as dehydroascorbic acid and diketogulonic acid. Cooling slowly before refrigeration resulted in an even greater proportional amount of the total appearing as diketogulonic acid. Baked and pressure-cooked potato tubers tended to respond similarly in respect to changes in the relative amounts of these components.

SUMMARY

The effect of two methods of cooking and of refrigeration after cooking on the ascorbic acid, dehydroascorbic acid, and diketogulonic acid contents of a number of potato varieties was investigated. Baking tests were carried out on three varieties (Washington Russet, Red Warba, and Triumph). Cooking in a pressure saucepan was tested on two varieties (Kennebec and Redcote).

Neither baking nor pressure-cooking changed significantly the reduced ascorbic acid content of the potato tubers under the conditions of this experiment.

Potatoes refrigerated for 24 hours after baking or pressure-cooking retained significantly less ascorbic acid than the freshly cooked product. For the baked potatoes the mean retention was 39% of the amount present immediately after baking. Pressure-cooked potatoes which were cooled quickly before refrigeration retained a mean of 45% and those which were allowed to cool spontaneously to room temperature before refrigeration, a mean of 36% of the ascorbic acid in the freshly cooked product.

All of the potato varieties tested contained appreciable amounts of dehydroascorbic acid before cooking. Both baking and pressure-cooking reduced significantly the dehydroascorbic acid content. For the baked potatoes the mean retention of this component was 34% and for pressure-cooked, 24% of the amount in the raw tubers. Refrigeration for 24 hours of the cooked product increased significantly the dehydroascorbic acid content. Pressure-cooked potatoes cooled quickly before refrigeration contained more of this component than did the baked and pressure-cooked potatoes which were allowed to cool spontaneously.

Small amounts of diketogulonic acid were present in the raw potato tissue. Baking or pressure-cooking increased significantly the content of this substance, with further increases on refrigeration of the cooked product. Pressure-cooked tubers cooled quickly before refrigeration contained less of this component than did baked and pressure-cooked potatoes allowed to cool spontaneously to room temperature, i.e., less dehydroascorbic acid was converted into diketogulonic acid with rapid cooling.

Not all of the decreases in ascorbic and dehydroascorbic acids in the cooked and refrigerated potatoes could be accounted for by corresponding increases in diketogulonic acid.

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EFFECT OF LOW TEMPERATURE, SODIUM NITRITE, SODIUM CHLORIDE, AND FORMALDEHYDE ON THE APYRASE SYSTEM OF THE MUSCULAR TISSUE[a]

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One of the enzyme systems most essential for living organisms is the apyrase system which hydrolyzes adenosine triphosphate (ATP). The ATP splitting mediates the phenomenon of movement, e.g. muscle contraction, constriction of the cell plasma on cell separation, separation of the chromosome deposits in the anaphase of the mitose, etc. *(13, 34, 35)*. This enzyme system seems to be essential for the transfer of energy obtained from metabolism to living structures *(30, 17)*. It has been found also in bacteria *(5)* and viruses *(10)*.

The reactions on which muscular contraction are based can be regarded as going on in two stages: the first step, producing about 10,000 cal/mole ATP *(30, 17)* and an inorganic phosphate, leads to adenosine diphosphate. In the skeleton musculature of the vertebrates this reaction is catalyzed mainly by the myosin-adenosine triphosphatase bound to the actomyosin, and partially by the magnesium-activated adenosine triphosphatase of Kielley and Meyerhof *(13)*. In the second step the adenosine diphosphate is dismutated to adenosine monophosphate and -triphosphate by the enzyme myokinase. In the physiological pH-range between 6 and 7 the musculature can be considered as a specific apyrase system *(28, 19)*.

The apyrase activity can easily be measured in cross-striated muscle tissue. The time-reaction curve for the ATP split by the musculature shows two different parts, each of which can be considered in approximation as a first order reaction *(21)*.

When ATP is added to a suspension of cross-striated muscle fibers in a suitable medium an irreversible contraction of the fragments is obtained *(16)*; this can be measured under the microscope *(22, 23, 24)*.

Extensive investigation of the apyrase system has revealed a close correlation between contraction and ATP splitting *(36)*. It was evident that the two methods for measuring enzyme activity and contractility might be used to investigate the effect of adjuncts and freezer storage on foods. In our opinion a food adjunct cannot be considered physiologically harmless if small quantities of it inhibit the apyrase system of fish or mammal muscular tissue irreversibly.

This investigation deals mainly with the effect of sodium nitrite, sodium chloride, and formaldehyde on the apyrase system of white muscle of carp. In order to support some views of general importance results of freezer storage experiments and artificial ripening of meat are briefly discussed too.

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[a] Dedicated to Prof. Dr. Dr. h.c. R. Plank to his 70th birthday on 6 March 1956.

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EXPERIMENTAL

Preparation of musclebrei for the enzyme mixture and for testing contractility. White muscular tissue of tench (Tinca vulgaris Cuv.) or carp (Cyprinus carpio L.) or Gastrocnemius musculature of beef poor in connective tissue was thoroughly minced in a meat grinder immediately after death. Six-gram samples sealed in test tubes were kept for 3 hr. at +4° C. and then stored at −24° C. In storage tests for simultaneous determinations of proteolysis the samples were stored at −3.5° C. and +10° C.

The available creatine phosphate and the ATP reproduced in the process of glykolysis by decomposition of glykogen are enzymatically split after 3 hr. storage of the musclebrei at +4° C. A small difference between the P-values found after acid hydrolysis (1-N HCl, 7 min. at 100° C.) and the direct determinable P remained constant during the freezer storage and amounted to about 100 γ/g. muscle. Probably this phosphoric acid ester represents a nucleotide which forms a stable complex with the structural protein actine *(25, 2)*.

Measurement of ATP breakdown in musclebrei. 0.5 g. (in some cases 1 g.) of muscular tissue were ground with 1 g. of sea sand, then washed into a 50 ml. flask with 25 ml. veronal buffer (pH 7) or veronal acetate buffer (pH 6.2 or 7). For testing food adjuncts the buffer volume was 23 ml. whereas the other 2 ml. contained the food adjunct. In standard operation 15 mg. of ATP-Na_4 salt (product of Zellstoff-Fabrik Waldhof, Wiesbaden) in 5 ml. of water were added at once; in case of food adjuncts, after wanted reaction time of the agents on the apyrase system. The purity of the ATP-salt was found to be over 80% by analysis of the inorganic-acid labile and the total P. The enzymatic ATP breakdown took place at 20° ± 0.5° C. and was stopped in due time, in general after 70 seconds, by addition of trichloroacetic acid, the end concentration of the acid amounting to 10%. After filtering, 2 aliquots of the filtrate were acidified to 1-N HCl and brought to equal volumes. One sample was hydrolyzed at 100° C. for 7 minutes. Determination of P was made with a modified method of Berenblum and Chain *(1)*. The yellow complex compound obtained by reaction with molybdic acid was extracted with butanol and reduced by stannous chloride to molybdenum blue. Phosphorus was colorimetrically determined with the ELKO II (Zeiss Co.) using the filter S 72. The extinctions are sufficiently constant in the interval from 30 to 90 minutes after color development. The values given for the enzymatic ATP hydrolysis are averaged from three parallel measurements.

When testing the action of $NaNO_2$, NaCl, and HCHO, ATP-splitting is represented as a percentage of the value obtained in the absence of food adjuncts at pH 6.2 and 7 (Table 1). The molarity of the Na-salts in the incubation mixtures in the absence of

TABLE 1

The influence of pH value and buffer on the ATP splitting

pH value	Buffer	% ATP split
6.2	veronalacetate	52.9
7.0	veronalacetate	61.4
7.0	veronal	73.9

preservatives and under use of the veronal acetate buffers pH 6.2 and 7 was 0.13 and for the veronal buffer pH 7 only 0.05.

In the trials to regenerate the apyrase activity of musclebrei, treated with formaldehyde, by addition of cysteine the method of Kuschinsky and Turba *(14, 15)* was applied.

Testing of contractility. One gram of musclebrei was homogenized in 18 ml. 0.16 M KCl solution or veronal buffer (pH 7) or veronal acetate buffer (6.2 or 7) for 15 seconds. One ml. of the relatively rough cut material—in case of addition of food adjunct after one hour's reaction time—was suspended in 50 ml. of the homogenizing solution. One drop of the suspension was transferred to a tissue culture chamber, 0.1 mm. in height, and covered with a slip glass which allowed lateral addition of ATP. Long shaped, non-contracted fiber fragments were focussed in the phase microscope. A small amount of solid ATP-Na_4 salt was added, so that the ATP concentration in

the suspension did not exceed 10^{-2} M. The beginning effect of ATP could be recognized by the appearance of a stream setting in with the dissolution of ATP. Changes in the length and width of the muscle fiber fragments were measured with the objective and ocular micrometer.

Analysis of ammonia-nitrogen in fish musclebrei. Five grams of NaCl, 5 g. of fish muscular tissue homogenized in 25 ml. water, 15 ml. ethanol (95%) and 5 ml. of sodium bicarbonate were put into a high wash bottle. After thorough shaking 1200 liters of air washed in diluted H_2SO_4 and KOH were passed through the solution. The volatile amines in the escaping air were collected in diluted H_2SO_4 which was back-titrated with 0.02-N NaOH.

Determination of free tyrosine-nitrogen in fish musclebrei. The free tyrosine-N was determined colorimetrically with the reagent of Folin and Ciocalteu *(9)*, specified by Wood, Sigurdsson, and Dyer *(37)*. The method is not specific in that tryptophane, phenols and SH-compounds give the molybdenum blue color with the reagent. We found that bacterial changes of the fish musclebrei can be followed quite well with the reagent.

pH determination in fish musclebrei. One gram of muscular tissue after grinding with 1 g. of sea sand was taken up with 25 ml. distilled water. After filtering the suspension, pH was measured with the glass electrode using the acidometer of Lange Co., Berlin.

RESULTS

Effect of low temperatures. Storage temperatures between 0° and –190° C. acting for several days do not damage the contractile mechanism of muscle fibers *(20, 23)*. The freezing velocity seems to have no influence on the contractility. No difference in behaviour of different muscle samples, whether frozen in stagnant air at –3.5° C. or by dipping in liquid air, was discovered. After storage for 3 months and longer at a temperature under –18° C. no change of the apyrase activity of beef and fish muscular tissue was found *(20)*. This result is astonishing because considerable denaturation occurs in actomyosin *(27, 6)* during such a long freezer storage. Actomyosin has been found to represent the main part of the contractile structures in the muscle, the fibrils. Both these findings apparently contradictory to each other become understandable by a discovery of Szent-Györgyi and his school *(31)*, who found that the myosin molecule can be split up into two components, the L- and H-meromyosin. H-meromyosin possesses the whole ATPase activity and practically all free SH-groups of the myosin. By freezing, only the L-meromyosin is denatured; the H-meromyosin stays intact.

It is known that the cold storage near 0° C. of food containing protein results in rapid and frequently more far-reaching changes than storage at temperatures below –10° C. *(29)*. Besides physico-chemical changes, bacterial contamination can play an important part at temperatures even below 0° C. during longer storage times. In storage experiments with musclebrei of carp and tench after a storage time from 30 to 45 days at a temperature of –3.5° C., we found repeatedly a distinct drop of the apyrase activity in the brei (Figure 1).

Storage results with the same sample material at +10° C. made it probable that bacterial processes in the actomyosin are not responsible for this change (Figure 2).

A comparison of Figure 1 and Figure 2 shows that the amount of the products of proteolysis, free tyrosine and ammonia, at +10° C. increased as expected in a few days and much higher than at a storage temperature

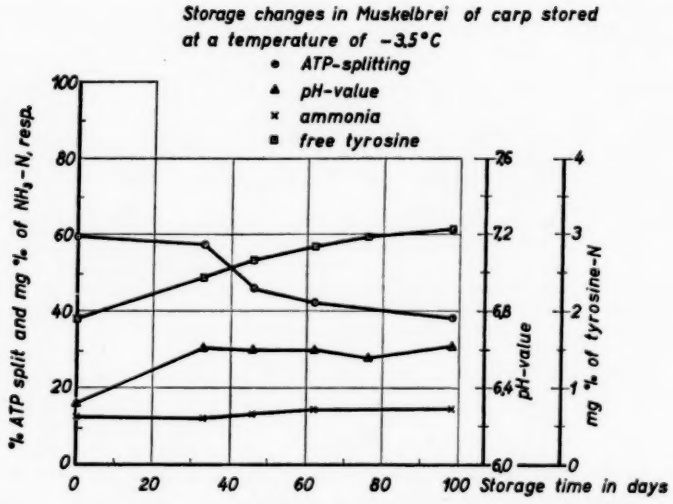

Figure 1. Storage changes in musclebrei of carp stored at a temperature of −3.5° C.

of −3.5° C. in a month. On the other hand, the apyrase activity in the fish muscle samples stored at +10° C., and already spoiled by bacterial activity, did not change significantly.

In studying the question of the irreversible changes of muscle structure as caused by freezer burn, experiments have been made with samples of red perch fillet; it has been found that the damaged muscle fibers

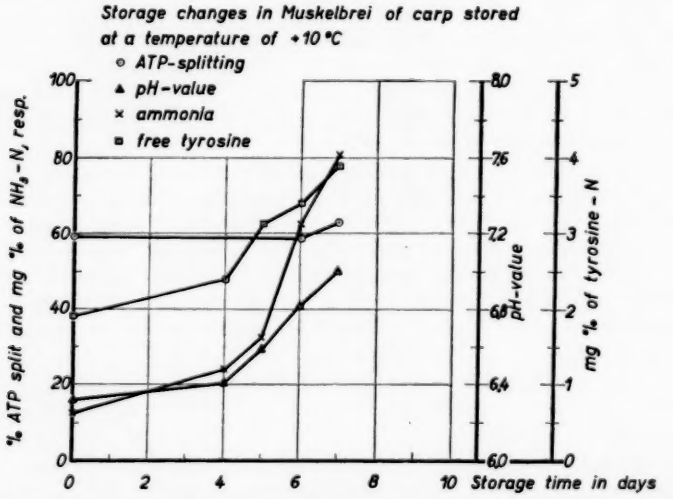

Figure 2. Storage changes in musclebrei of carp stored at a temperature of +10° C.

located in the freezer burn zone no longer contracted after ATP addition *(22)*. Obviously a relation exists between the contractility and fine structure maintenance in muscle fiber fragments.

Experiments with musclebrei of carps and tenches stored for more than 30 days at –3.5° C. gave the same results. After a short homogenizing of the brei besides fragments of good conserved fine structure, many muscle fiber fragments of heavy altered, blistered structure were present. Although the intact fibers again showed good contractility, the destroyed fragments were found not to contract any longer *(23)*. As noted above, the apyrase activity in this brei is distinctly lower than at the beginning of storage (Figure 1).

In testing papain-containing enzymes for artificial meat ripening identical relations were encountered *(24)*. Experiments started on the effect of high dosages of Roentgen rays on muscle tissue point in the same direction.

In the course of normal meat ripening the fine structure of the muscle fibers visible under the microscope apparently is not disorganized. Consequently, normal meat ripening does not lead to a loss in contractility of the muscle fibers. *Rigor mortis* as known can be interrupted by the "plasticizing" effect of ATP.

Considering enzymatic and bacterial attack *(18)* on muscle fibers, it seems to be valid in general that actomyosin is relatively stable.

The close correlation between ATP splitting, contractility, and fine structure does not exist if by any treatment the muscular system is fixed, e.g. by action of sufficient high formaldehyde concentrations.

The Action of $NaNO_2$, NaCl and HCHO on ATP-splitting

Sodium nitrite. Figure 3 shows the measured values of the ATP split in musclebrei for different nitrite concentrations. The concentrations of

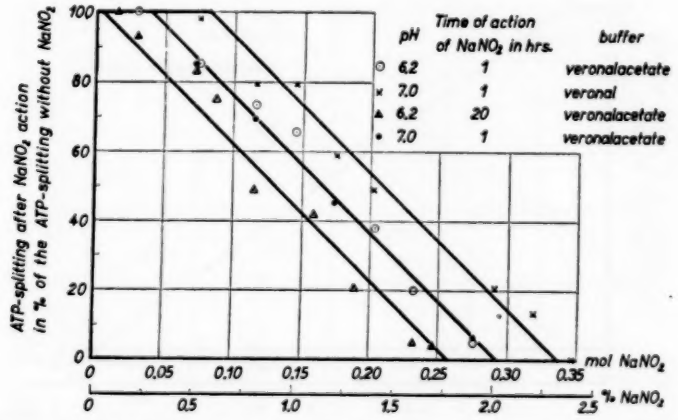

Figure 3. The influence of the sodium nitrite concentration on the ATP splitting of musclebrei of carp at a temperature of +20° C.

nitrite necessary to obtain the same inhibition of the ATP breakdown are higher with the veronal buffer (pH 7) than with the veronal acetate buffer (pH 7). Further it can be seen that a given nitrite concentration yielded the same amount of ATP breakdown whether veronal acetate buffer of pH 6.2 or 7 was used. Hence, it follows that in the pH range between 6.2 and 7 the pH value is of no influence on the inhibition of the apyrase activity by nitrite in the musclebrei. The ionic strength related to Na^+ is much more decisive (see p. 52). A small but distinct increase in the effect of nitrite results at pH 6 (veronal acetate buffer) from an action of 20 hours as compared with an action of one hour.

It is remarkable that only a concentration of about 2% sodium nitrite prevents the ATP splitting completely. Sodium nitrite therefore is no specific inhibitor of the apyrase system. The nitrite is more effective on other enzyme systems, and in these cases, the pH value under predominant participation of the nitrite ion might be of importance *(32, 8, 7, 3, 4)*.

Our results make it probable that those groups in actomyosin essential for the ATP splitting are relatively indifferent to the nitrite ion.

Sodium chloride. The effect of different NaCl concentrations on the ATP splitting at pH 6.2 after an action time of one hour is illustrated in Figure 4. NaCl is somewhat less effective than $NaNO_2$ as under our test conditions only a molarity of about 0.38 (2.2%) leads to an almost complete inhibition of the apyrase system. Hence, we may conclude that the anions different in NaCl and $NaNO_2$ also participate in the inhibiting effect on the muscular apyrase system to a small degree.

Because of the relatively high amount of sodium chloride present in the electrolytes of the body fluids of higher animals, a specific inhibition of the apyrase system by means of sodium chloride could not be expected.

Formaldehyde. The apyrase system is inactivated to a larger degree by the same amount of formaldehyde when the pH value is lowered from the neutral point to pH 6.2 (Figure 5). This result is in accordance with

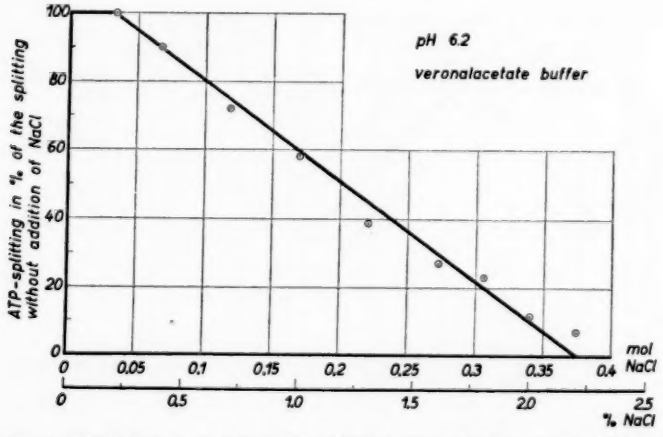

Figure 4. The influence of the sodium chloride concentration on the ATP splitting of musclebrei of carp at a temperature of +20°C.

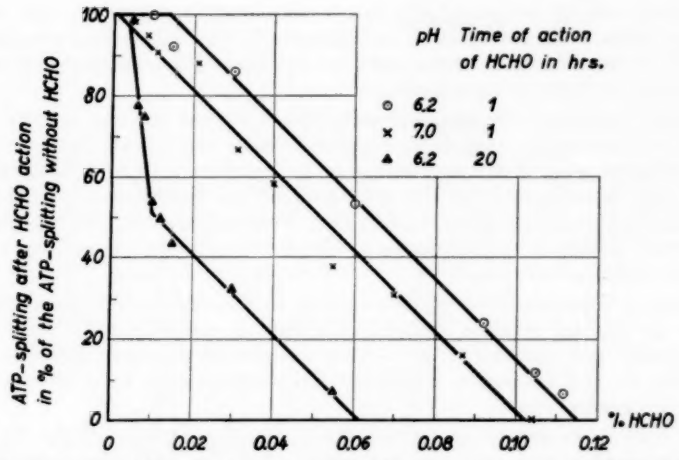
pH Time of action of HCHO in hrs.
6.2 1
7.0 1
6.2 20
ATP-splitting after HCHO action in % of the ATP-splitting without HCHO
% HCHO
0 0.02 0.04 0.06 0.08 0.10 0.12
0 20 40 60 80 100

Figure 5. The influence of the formaldehyde concentration on the ATP splitting of musclebrei of carp at a temperature of +20°C.

observations for the behaviour of formaldehyde in tanning hides. However, the single groups of the protein reacting with formaldehyde react differently, depending upon the H^+-concentration *(33)*.

To gather additional information on the inactivation by formaldehyde as a function of time (Figure 5), measurements of the ATP breakdown of carp muscle tissue treated with 0.02% and 0.05% formaldehyde at pH 6.2 have been carried out. As Figure 6 shows, the inhibition of the muscular apyrase system obtained by a higher formaldehyde concentration is

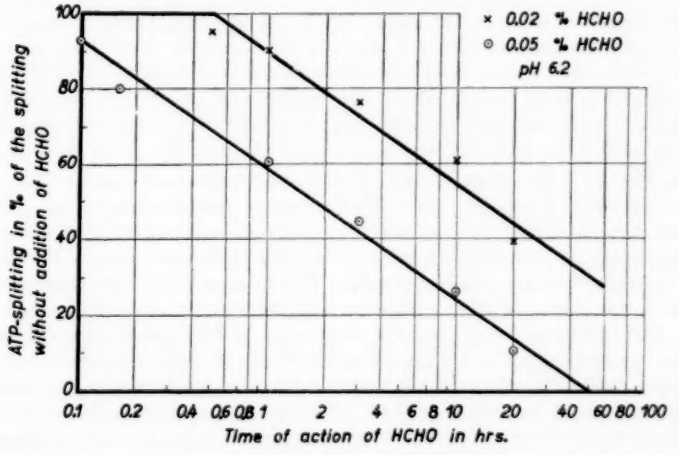
0.02 % HCHO
0.05 % HCHO
pH 6.2
ATP-splitting in % of the splitting without addition of HCHO
Time of action of HCHO in hrs.
0.1 0.2 0.4 0.6 0.8 1 2 4 6 8 10 20 40 60 80 100
0 20 40 60 80 100

Figure 6. The influence of action time of formaldehyde on the ATP splitting of musclebrei of carp.

completed also by an essentially lower concentration; however, the completion takes a longer time. This elementary but substantial statement should be considered in discussing the formaldehyde concentration still admissible for preserving purposes.

Thus formaldehyde is a strong inhibitor of the apyrase system. It would be conceivable that formaldehyde attacks the myosin in a definite and essential part of the molecule, e.g. by reaction with free SH-groups. This view is supported by the inhibition of the bacteriostatic action of formaldehyde by cysteine *(38, 39, 40)*. Furthermore the ATP splitting of myosin poisoned by Salyrgan—a SH-poison—can be restored by addition of cysteine *(14, 15)*.

Tests to regenerate the apyrase activity in formaldehyde treated muscle tissue by adding cysteine, failed. This finding coincides with results of Kuschinsky and Turba *(14, 15)*. They discovered that formaldehyde—in contrast to the reversible inhibiting SH-poisons—acts as a denaturing agent on actomyosin.

It may be assumed that formaldehyde showing a great affinity for the different end groups of proteins reacts with free SH-groups, perhaps under formation of no longer reactive thioethers *(26)*. At the same time or later, a series of irreversible reactions will take place with cross-linking of protein chains and micelles by methylene bridges in other parts of the molecule. Recently, these and other possible types of reaction have been discussed *(33)*.

This presentation of the formaldehyde effect leads to the assumption that even formaldehyde cannot be regarded as a specific inhibitor of the apyrase system.

The Action of $NaNO_2$, NaCl and HCHO on Contractility

It is known that stretched muscle fiber fragments contract in diluted alkalichloride solution after ATP addition. A 0.16 molarity is held as optimal concentration, which is also encountered in the serum of warm blooded animals. Preliminary tests showed the contractility to exist for the same extent in both buffers used. The average of the muscle shortening in longitude in the absence of food adjuncts was 53% with veronal buffer (pH 7) and 54% with veronal acetate buffer (pH 6).

In Figure 7 the mean values of the contraction of muscle fiber fragments obtained from at least 10 individual measurements are plotted against the inhibition of the ATP breakdown realized by the compound under study. It can be seen that the contractility increases with decreasing concentration of food adjuncts. The contractility decreases rapidly in the lower concentration range and changes more slowly in the higher one. The ATP splitting, on the contrary, is lowered in approximately linear measure with a rising concentration of different food adjuncts (Figures 3-5). Based on the results of the measurements of contractility of the fibrillary proteins, sodium chloride and sodium nitrite prove to be much more harmless than formaldehyde, even at the same degree of inhibition of the ATP splitting. This is especially true initially when the concentration of inhibiting substance is low.

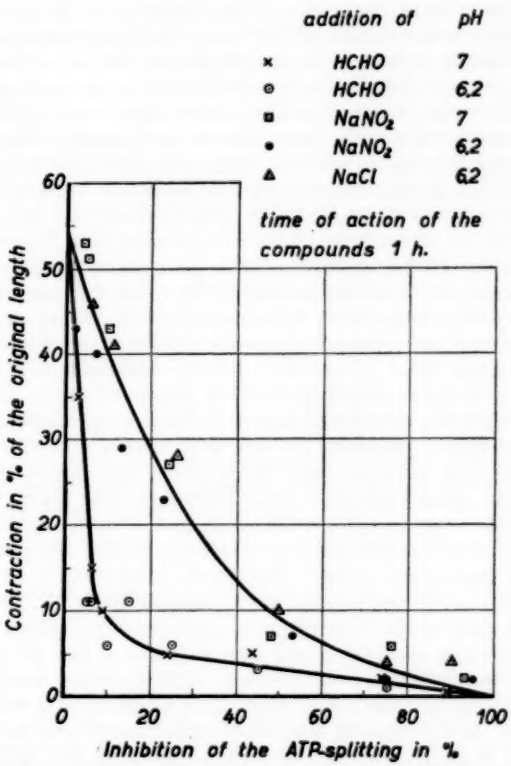

Figure 7. The influence of the inhibition of the ATP splitting by several adjuncts on the contraction of muscle fiber fragments.

DISCUSSION

Testing the physiological effect of food adjuncts on the human body is still linked with difficulties. An essential but limited evidence of harmlessness can be gathered from feeding trials on rats or other mammals conducted over many generations. This holds also for conclusions drawn from the results of enzyme inhibiting tests with food adjuncts. It could be reasoned that the components of orally administered food behave quite differently against enzymes distant from the digestive system compared with the same compounds injected or in any way directly reacting with the enzymes present in other organs. Further, the enzyme system maximally influenced by the food adjunct will frequently not be chosen.

If food adjuncts with concentrations close to those needed for preservation purposes cause a strong and irreversible inhibition of vital enzyme systems like apyrase, the corresponding test results may serve for a negative judgment of the agent in question.

Such compounds, when incorporated with food, might inhibit the adenosinetriphosphatases of the cells of the alimentary canal. Though

apyrase systems of musculature and other tissues may distinguish in some respects, two important results of Hoffmann-Berling point to this danger: (a) The contractile protein of undifferentiated cells acts like actomyosin as an adenosine triphosphatase and is inhibited by the same poisons *(11)*. (b) The fundamental movements of cells are influenced by chemical compounds and temperature in the same manner as muscular contraction *(12)*.

In our opinion an admission of 0.001% formaldehyde to food could be regarded as dangerous, because with a tenth-fold higher formaldehyde concentration (0.01%) the ATP in the muscle tissue will be split to about 50% only after an action time of 20 hours (pH 6.2) (Figure 5).

The inhibition of the apyrase system by sodium nitrite starting only at relatively high nitrite concentrations (0.2%) does not permit the assumption that the nitrite may be as harmless as NaCl. There might be other enzymes which are most sensitive against nitrites in acid medium.

The inhibiting effect of sodium chloride and sodium nitrite on the apyrase system is apparently stipulated by the alkali ion in the first place. It is known that the structural proteins dissolve in alkali salt solutions above 0.45 molarity. Recently it has been shown that ATPase of the actomyosin-gel acts stronger and under other conditions than does the ATPase of the actomyosin-sol *(34)*. Therefore, the effect of NaCl and $NaNO_2$ probably represents reversible processes related to the sol-gel-transformation of the structural proteins. Contrary to that the effect of formaldehyde might lead to irreversible changes of the protein.

The simple contraction method might become useful in other fields, too. For the first orientation into physical and chemical effects of unknown nature on muscular tissue and proteins in general, say as a preliminary test, it is recommended that the effect be applied at the muscle fiber fragments and contractility be tested after ATP addition. In many cases concentrations or dose ranges of beginning effectiveness can be estimated in this way. If it is of interest, the measurement of the ATP breakdown could be brought up for more precise evidence.

SUMMARY

The effect of sodium nitrite, sodium chloride and formaldehyde on the apyrase system of the white carp muscle was investigated. Besides the inhibition of the ATP splitting, the contractility of the muscular fibers treated with the additives was tested by adding ATP-salt.

Sodium nitrite was found not to be a specific inhibitor of the muscular apyrase system. In the pH range between 6 and 7 the sodium nitrite effect was independent of the pH value and primarily subject to the Na-ion concentration.

The effect of sodium chloride corresponds in magnitude to the nitrite effect. Obviously, the anion, too, participated to a small degree in the inactivation.

Formaldehyde showed a much stronger inhibiting effect than the alkali salts studied. A concentration of about 0.1% formaldehyde (pH 7) under our conditions, was found to inactivate completely the muscular apyrase system. The inhibition effect obtainable by higher formaldehyde concentration was also realized with a lower concentration but prolonged reaction time.

The contractility of muscle fibers treated with rising concentrations of the tested adjuncts first showed a sharp decline and then slowly to approach zero value at the concentration of the adjunct, which inhibited completely the apyrase activity. With formaldehyde the abrupt drop in contractility was more distinct than with sodium nitrite or chloride.

The apyrase system of the cross-striated muscular tissue of beef and fresh water fish was extraordinarily stable against different influences, like low temperature, autolysis, and microbial activity. After storage for 30 to 45 days at –3.5° C. a decrease of the apyrase activity in musclebrei of fresh water fish was observed.

It has been proved that the disorganization of the fine structure of muscle fibers is correlated with the loss of contractility and the decrease of apyrase activity. Therefore, the loss of contractility can be used as a test for an inhibitive effect of food adjuncts on the apyrase system.

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THE CAROTENOIDS OF RUBY RED GRAPEFRUIT

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The carotenoids of grapefruit, in particular pink or red varieties, have been the subject of several investigations. Matlack *(9)* in 1928 reported that the principal pigment of the Indian Red pummelo and the pink grapefruit was lycopene. Later *(10)* he confirmed the presence of lycopene in the Indian Red pummelo *(Citrus grandis* Osbeck), and *(11)* obtained lycopene and β-carotene in crystalline form from Foster and Marsh pink grapefruit *(Citrus paradisi* Macf.). These two pigments were reported from Ruby Red grapefruit by Huffman, Lime, and Scott *(5)*. Khan and Mackinney *(7)* investigated the pigments of white, pink, and red grapefruit, and reported a total of 11 bands on chromatography; in the pink strains β-carotene and lycopene predominated, with lesser amounts of zeta-carotene and phytofluene, and traces of α- and γ-carotenes. In view of the complexity of the carotenoid mixtures from oranges *(1, 2)* and tangerines *(1)*, and the absence of any previous reports of any xanthophyll in grapefruit, the carotenoid pigments of Ruby Red grapefruit were investigated by the use of countercurrent distribution to separate the carotenoids into hydrocarbon, monol, and diol (plus polyol) fractions which were then further fractionated by chromatography. The carotenoids in the peel were also studied; these apparently have not been previously investigated. By this means the presence of over 20 carotenoid constituents was shown. Lycopene, β-carotene, zeta-carotene, phytofluene, and phytoene were the main constituents; most of the xanthophylls found in oranges and tangerines occurred in Ruby Red grapefruit as minor constituents.

EXPERIMENTAL

The Ruby Red grapefruit used in these experiments originated at Mission, Texas, and was purchased at a local market in early March, 1955. The fruit was hand-peeled and the peels and peeled fruit (pulp) extracted separately. The peels amounted to 21% by weight of the whole fruit.

The peeled fruit was blended with an equal weight of water in the presence of an excess of calcium carbonate; an equal volume of methanol was then added and the mixture stored at 2° C. overnight. The mixture with filter aid added was filtered in Buchner funnels precoated with filter aid and the filter cake, after washing with water-methanol (1 + 1 by volume), was extracted with acetone in sintered glass funnels. The extracts were worked up as previously described *(1)*, including saponification.

The peels were covered with methanol and allowed to stand overnight at 7° C. The methanol was decanted off, the peels blended with acetone, filtered on sintered glass funnels, and the filter cake washed with acetone until the washings were apparently colorless. The extracts were worked up as with the peeled fruit.

The total carotenoid content of the saponified extract from the peeled fruit was equivalent to 8.2 mg./kg. (measured as β-carotene); the corresponding figure for the peels was 10.4 mg./kg. It was calculated that 74% of the carotenoids (as β-carotene) were present in the peeled fruit.

Countercurrent distribution runs were made in a glass Craig apparatus with the saponified carotenoids from the pulp and the peel; the solvent system petroleum ether–

99% methanol was used, and 97 transfers were made. The color in each tube of the apparatus was then measured in an Evelyn photoelectric colorimeter, with filter 440, and the results calculated as β-carotene *(1)*. The contents of the tubes were then combined into fractions, which in solution in petroleum ether (hydrocarbon and monol fractions) or benzene (diol plus polyol fraction) were then chromatographed on columns of magnesia (Westvaco No. 2642) plus filter aid (1 + 1 by volume). Spectral absorption curves were run in a Cary recording spectrophotometer. Eluents used with the hydrocarbon fraction were sucessively 14, 20, 30, 45, and 70% benzene in petroleum ether; benzene; 3, 5, 7, and 10% acetone in benzene; and finally 7% ethanol in petroleum ether. With the monol fraction the eluents used were 0.5, 0.7, 1, 1.4, 2.5, 5, 7, and 10% ethanol in petroleum ether. The eluents used with the diol plus polyol fractions were successively 3.5, 5, 7, 10, 15, and 25% ethanol in petroleum ether, and finally 20% ethanol in benzene.

RESULTS AND DISCUSSION

The results obtained on countercurrent distribution are given in Table 1. In both the peel and pulp carotenoids, the great majority of the pigments were hydrcoarbons, but small monol and diol (plus polyol) fractions were obtained in both cases; greater percentages of both monols and diols (plus polyols) were obtained from the peel carotenoids, and a definite monoether monol fraction was obtained. This fraction was previously found to be more prominent in both orange and tangerine peels *(3, 4)* than in the corresponding pulp carotenoids. The distribution curve for the peel carotenoids is given in Figure 1; the positions of the maxima were in good agreement with those obtained in corresponding runs with oranges and tangerines. Due to the small amount of material in the diol (plus polyol) fraction, counter-current distribution runs with the system benzene-petroleum ether-87% methanol, by means of which the diol (plus polyol) fraction can be separated into 4 fractions, were not made.

The constituents obtained on chromatographing the 3 fractions of the pulp carotenoids, and the 4 fractions of the peel carotenoids are given in Table 2. The monol and monol monoether fractions of the peel carotenoids were combined, since they were not cleanly separated. In most cases the spectral absorption maxima were similar to those previously published for orange carotenoids *(1, 2)*; hence these values were omitted from Table 2 but are given for a few of the constituents which had not previously been found in oranges in Table 3. The approximate percentages in Table 2 were obtained by calculating the total absorbance of each constituent at the maximum second highest in wave length. In calculating the percentages, the values were corrected for the differences in the absorbancy index (1 mg./ml. in a 1 cm. cell). The values were used as follows: phytoene 85

TABLE 1

Countercurrent distribution of saponified carotenoids from Ruby Red grapefruit pulp and peel

	Approximate percentage of total carotenoids (measured as β-carotene)	
	Pulp	*Peel*
Hydrocarbons (I)	93	76
Monols (II-A)	3	7
Monoether monols (II-B)	....	3
Diols and Polyols (III)	4	14

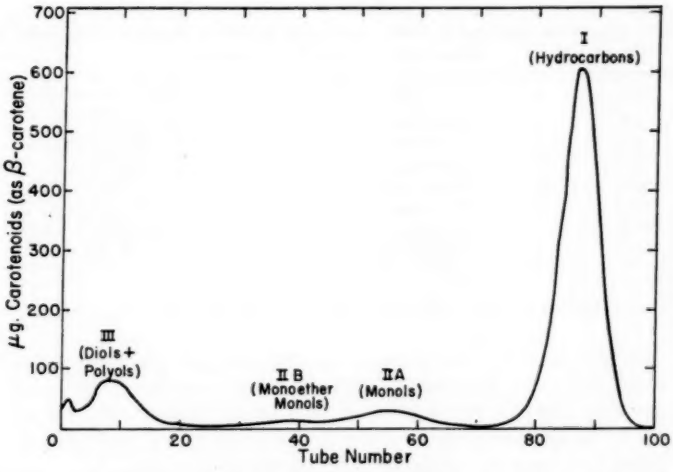

Figure 1. Countercurrent distribution of saponified carotenoids from Ruby Red grapefruit peel. System petroleum ether—99% methanol.

TABLE 2

Carotenoids obtained from Ruby Red grapefruit peel and pulp

Fraction	Constituent	Approximate percentage in total carotenoids	
		Pulp	Peel
I-1	Phytoene	16	47
I-2	Phytofluene	4.4	14
I-3	α-Carotene	....	0.1
I-4	β-Carotene	27	7.2
I-5	Phytofluene-like	....	0.4
I-6	Zeta-Carotene	3.5	7.2
I-7	γ Carotene-like	0.8	0.4
I-8	Lycopene	40	11
II-1	Substance 377	0.2	....
II-2	Substance 335	0.6	0.5
II-3	"Hydroxy-α-Carotene"	0.2	0.1
II-4	Cryptoxanthin	0.7	1.4
II-5	Cryptoxanthin-like	0.4	....
II-6	Cryptoflavin-like	....	1.3
II-7	Rubixanthin-like	0.5	0.3
II-8	Cryptochrome-like	....	0.2
III-1	Lutein	0.3	0.9
III-2	Antheraxanthin	0.7	....
III-3	Violaxanthin (+Zeaxanthin?)	0.9	1.0
III-4	Luteoxanthins	0.4	1.8
III-5	Mutatoxanthins	0.4	0.2
III-6	Valenciachromes	0.2	0.3
III-7	Trollichrome-like	....	0.2
III-8	Auroxanthins	0.3	1.6

TABLE 3

Spectral absorption maxima of some carotenoid fractions obtained from Ruby Red grapefruit not found in oranges

No.	Constituent	Maxima, in petroleum ether, mμ
I-5	Phytofluene-like[1]	368,347,331
I-7	γ-carotene-like[2]	(492)[3],457,430
I-8	Lycopene[2]	504,473,436
II-1	Substance 377[2]	377,357,337
II-2	Substance 335[2]	335,320,306
II-5	Cryptoxanthin-like[2]	478,452,(428)[3]
II-7	Rubixanthin-like[1]	489,457,431

[1] Pulp.
[2] Peel.
[3] Values in parentheses represent humps or shelves on spectral absorption curves.

(13), phytofluene 146 (the average for the cis and all-trans forms) *(8)*, α-carotene 273 *(15)*, β-carotene 258 *(15)*, zeta-carotene 204 *(12)*, and lycopene 346 *(14)*. The indices of the other minor constituents were assumed to be the same as β-carotene, except for two fractions (I-5 and II-1, Table 3) where the value for phytofluene was used.

The results for the pulp carotenoids confirm earlier work that lycopene and β-carotene were the principal constituents, and confirm Khan and Mackinney's report *(7)* of significant amounts of phytofluene and zeta-carotene. Phytoene was also found to be a major constituent; it had apparently not been reported previously in grapefruit where it amounted to nearly half of the peel carotenoid mixture. A trace of α-carotene was found in the peel only, and minor fractions which appeared to be impure γ-carotene were obtained from both pulp and peel. In the peel carotenoids a minor, phytofluene-like band was found on the column between β- and zeta-carotenes. The ratio of lycopene to β-carotene was around 1.5 for both pulp and peel carotenoids.

As was the case with orange and tangerine peel carotenoids *(3, 4)*, the monol fraction was found to be somewhat more complex in the peel than in the pulp carotenoids. In the pulp "hydroxy-α-carotene," cryptoxanthin, another band higher on the column (II-5) which resembled cryptoxanthin closely, and a rubixanthin-like fraction were found. Two colorless constituents, not previously found in oranges or tangerines were present, one (II-I) with highest wave length of 377 mμ, and the other (II-2) 335 mμ, in petroleum ether. Both of these constituents had 3 peaks on the spectral absorption curve. Fraction II-2 occurred together with "hydroxy-α-carotene"; the spectral absorption curve is given in Figure 2. The highest wave length peak was much higher than the second. The spectral absorption curve of II-2 is unlike that of any of the well-known carotenoids; it may belong to another class of compounds. A similar fraction was found in the peel carotenoids. Because it occurs in both pulp and peel carotenoids, it is probably not a peel oil constituent.

The peel carotenoids of the monol fraction differed from those of the pulp in containing cryptoflavin-like and cryptochrome-like (spectral absorption maxima in petroleum ether 426, 400, 376 mμ) fractions, as was the case with orange and tangerine peels also *(3, 4)*. The former is chiefly responsible for the monoether monol (II-B) peak on the countercurrent

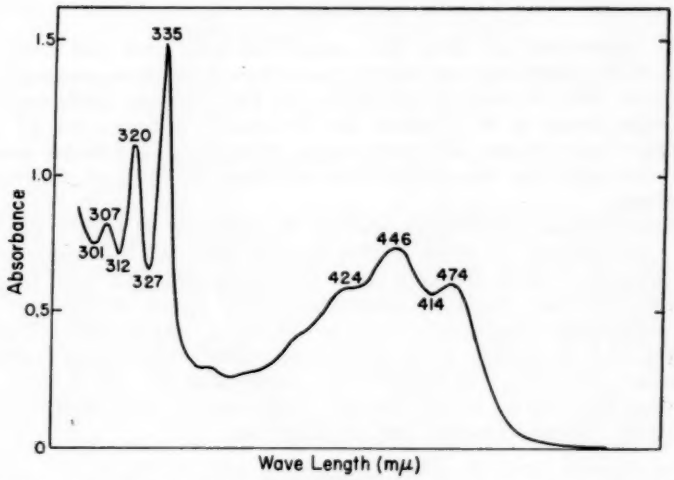

Figure 2. Spectral absorption curve of substance 335 (and "hydroxy-α-carotene") from Ruby Red grapefruit pulp (in petroleum ether).

distribution curve (Figure 1). Cryptoflavin and cryptochrome are the mono- and di-furan-oxides of cryptoxanthin *(6)*.

The chromatography of the diol + polyol fraction was not very satisfactory, due in part to the small amount of material available, and to the difficulty of separating this fraction by chromatography alone *(2)*. It is almost certain that some of the components of this fraction were overlooked. The results indicate that this fraction contained most or all of the numerous constituents found in this fraction in oranges *(2)* and tangerines *(4)*. One band (III-3) probably contained all-trans and cis-violaxanthins *(2)* and zeaxanthin, which in previous work have been found to separate poorly under the conditions used for chromatography. Also, the presence of auroxanthins was a strong indication that violaxanthin was also present, since violaxanthin is the parent substance which is transformed, via the luteoxanthins, to auroxanthins by means of acid. The valenciachromes *(2)*, which are furanoxides having a greenish fluorescence on the column similar to that of phytofluene, are more readily detected than the very pale colored valenciaxanthin, the parent epoxide. Hence the finding of the valenciachromes makes it very probable that valenciaxanthin was also present. As stated earlier, due to the small percentage of the diol plus polyol fraction in the total carotenoids, the countercurrent distribution system, by means of which this fraction can be separated into 4 parts, was not used.

As in work with orange and tangerine peels *(3, 4)* the spectral absorption curves of a number of the constituents of fraction III had very high peaks in the region 300-330 mμ. In order of elution these peaks were at 313, 319, 328, 308, and 320 mμ, in benzene. These peaks may be caused by the presence of residual substances from the peel oil having 3 or 4 conjugated double bands.

SUMMARY

The carotenoids of Ruby Red grapefruit pulp and peel have been examined by countercurrent distribution followed by chromatography. In accordance with previous investigations, the two principal pigments of the pulp were found to be lycopene and β-carotene, accompanied by lesser amounts of phytofluene and zeta-carotene. Phytoene was a major constituent of the pulp, and was the leading constituent of the peel, followed by phytofluene.

Countercurrent distribution showed the presence of both monols and diols (plus polyols), in much smaller amounts than the hydrocarbons. On chromatography these fractions from both pulp and peel were found to be rather complex, about 13 constituents being found in both pulp and peel carotenoids. Apparently most or all of the xanthophylls found in oranges and tangerines were present as minor constituents, including "hydroxy-α-carotene," cryptoxanthin, cryptoflavin-like (peel only), rubixanthin-like, lutein, antheraxanthin (pulp only), luteoxanthins, mutatoxanthins, valenciachromes, and auroxanthins.

Acknowledgment

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FACTORS AFFECTING THE DISPERSIBILITY OF "INSTANTLY DISSOLVING" DRY MILKS[a]

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Recent expansion in sales of nonfat dry milk through the introduction of several "instantly dissolving" dry milks has focused interest on the factors affecting the dispersibility of milk powders. In their thorough review Coulter, Jenness, and Geddes *(1)* indicate the limitations in our knowledge of this subject. Because "instantly dissolving" dry milks have recently come into production and because some if not all have been developed empirically, there is no information available on factors affecting their dispersibility. Considering that increased dispersibility is the essential advantage of "instant" dry milks the importance of elucidating the physical and chemical factors contributing to this dispersibility is obvious.

It is the purpose of this research to define chemical changes in the lactose and protein, physical changes in the size and shape of the particles, and the distribution of salts, lactose and protein which can account for the increased dispersibility of "instant" dry milks.

EXPERIMENTAL

Nonfat dry milk samples. Samples of spray-dried nonfat milk and instant dry milk No. 1 which was made by conversion *(2, 3)* of this spray-dried milk were obtained directly from an industrial production plant and an experimental processing facility. Instant dry milks No. 2 and 3 were purchased locally. Instant dry milks No. 1, 2 and 3 represented the 3 major instant dry milks on the retail market in 1954-55.

Demineralized instant dry milk No. 1 was prepared by passing skim milk through ion exchange columns before spray-drying and subsequent conversion in an experimental processing facility. Instant dry milk No. 1 used in the powder washing studies was composed of 53.8% lactose, 36.1% protein, 1.28% chloride, 0.46% sodium, 1.4% potassium, and 1.2% calcium.

Dry milk washing apparatus. The Buchner funnel (Figure 1), A, was fitted with a stainless steel ring, C, of approximately 6-inch outside diameter and 3-inch inside diameter, containing four 1½-inch threaded studs spaced equally around the ring about ¾-inch from the edge. The ring was completely covered with 80 mesh screen which had been soldered on. Rubber gaskets and spacer rings, D, were used to vary the volume of the powder chamber. Eighty mesh screen was soldered onto the bottom of the ⅛-inch thick top-most ring, E. A thin-walled steel cylinder, F, about 2 inches high was closely fitted inside the hole of the top ring. Any space between cylinder and top ring, or between bottom ring and funnel wall, was simply and effectively sealed with caulking tape. In use, the powder chamber formed by the holes in the spacer rings was loosely filled with the dry milk being washed. If ordinary powder is being examined, it is advisable to mix it with about half its weight of Celite to prevent channeling and cracking. A piece of 9 cm. Whatman No. 54 filter paper, G, is coated around the edge with vaseline and centered over the powder and rings. The uppermost ring is placed screen down over the filter paper and the entire assembly secured to the bottom plate

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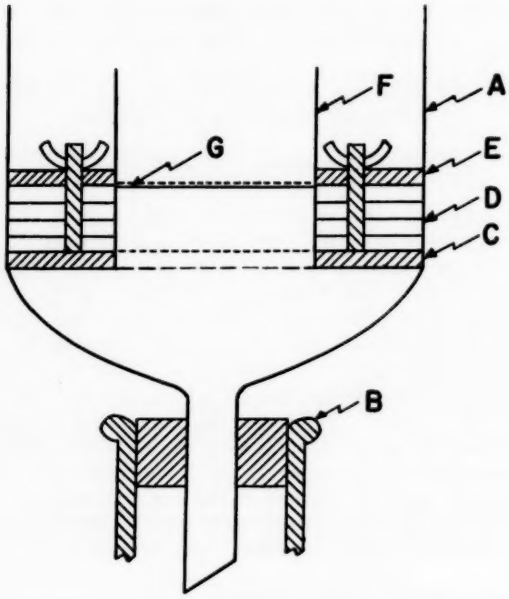

Figure 1. A cross-section view of the dry milk washing funnel.

with wing nuts. The Buchner funnel was connected by a 500-ml. filter flask, B, to a large evacuated tank which was used as the source of instant high vacuum of 20 to 30 mm. A convenient volume of water was placed in the cylinder. The filter paper prevented immediate contact of water and the dry milk until the stopcock between the evacuated chamber and the suction flask was quickly opened. The high, sudden vacuum pulled the water through the powder in a few seconds. The washings and residue were each made up to volume and analyzed for ions, lactose, and proteins. The weight of the dry milk washed off was determined by evaporating an aliquot of the washings.

Dispersibility time. Five grams of milk powder was dropped through a powder funnel into a 500 ml. Erlenmeyer flask containing 100 ml. water. The time lapse between the initial contact of the particles with the water and the complete submergence of the particles beneath the water surface was taken as the disperibility time. In this paper the terms dispersibility and wettability have been used somewhat interchangeably.

Protein nitrogen. Protein nitrogen was divided into casein nitrogen, soluble whey protein nitrogen, and lactalbumin and lactoglobulin protein nitrogen as described by Rowland *(4)*.

Chlorides. Ten ml. of silver nitrate reagent was added to an appropriate volume of sample and the excess silver back-titrated with standardized potassium thiocyanate solution. The silver nitrate reagent was prepared by mixing silver nitrate, 4.791 g., concentrated nitric acid, 200 ml., and 300 ml. of a saturated solution of ferric ammonium sulfate and adjusting the volume to 1000 ml. One ml. of this reagent equals 1 mg. chloride ion. In calculating the chloride concentrations in the surface layer of various particles it was assumed that the smaller particles are spheres and the larger particles are aggregates of spheres sharing only a small common surface.

Total lactose. Twenty-five ml. of Benedict's quantitative reagent, equal to 67.8 mg. of lactose hydrate was mixed with 3 to 5 g. of anhydrous sodium carbonate and titrated at the boiling point. The reagent was prepared as follows: Copper sulfate pentahydrate, 18.0 g., sodium citrate dihydrate, 200 g., potassium thiocyanate, 125 g., and anhydrous

sodium carbonate, 100 g., were dissolved separately and the solutions mixed together. Five ml. of 5% potassium ferricyanide solution was added and the volume adjusted to 1000 ml.

Alpha and beta lactose. Alpha and beta lactose were determined by the method of Sharp and Doob *(5)*.

Sodium and potassium. The sample was ashed at low temperature in the presence of sulfuric acid and the ions were determined using the Baird Associates Flame Photometer.

Calcium. The ashed sample was buffered at pH 4.4-4.6. Calcium was precipitated as the oxalate and titrated with potassium permanaganate.

RESULTS AND DISCUSSION

State of the lactose. Microscopic examination of the instant dry milk shows that the individual particles consist of large aggregates of smaller particles. These aggregate particles were birefringent in polarized light presumably because of the presence of crystalline lactose hydrate. A variety of dry milks was examined by the method of Sharp and Doob *(5)* for the distribution of alpha and beta lactose forms. Data in Table 1 show

TABLE 1

Distribution of alpha- and beta-lactose in milk powders

Sample	Lactose	
	% Alpha	% Beta
Spray-dried nonfat milk	40	60
Instant dry milk No. 1, A	60	40
Instant dry milk No. 1, B	58	42
Instant dry milk (demineralized aggregate, non-wettable)	56	44
Instant dry milk No. 2	40	60
Instant dry milk No. 3	42	58

that all of the instant dry milks of type No. 1 which were produced by a conversion of spray-dried nonfat milk have 60% alpha lactose in the final product as would be expected for a dry milk produced by slow evaporation of water. As is well known, the spray-dried nonfat milks contain 40% alpha lactose. The instant dry milks No. 2 and No. 3 also contain approximately 40% alpha lactose indicating that their preparation probably consists of spray drying. Although the high content of alpha lactose in instant dry milk No. 1 might be responsible for its rapid wettability by offering a more suitable surface for orientation of water molecules, this hypothesis could not explain the rapid wettability of instant dry milks No. 2 and No. 3.

Changes in protein. When instant dry milk No. 1 was heated, it browned at a more rapid rate than the spray-dried nonfat milk from which it was made. Further, the browned instant dry milk No. 1 was more fluorescent when analyzed by the technique of Tarassuk *(6)*. These observations suggested that a compound of casein and lactose may be formed via an active carbonyl-amine browning mechanism during the conversion process. This casein-lactose compound might be more wettable than casein itself, and hence affect the dispersibility of the instant dry milk. If such a combination of casein and lactose took place via the classical browning mechanism, a direct measure of this combination would be

TABLE 2

Protein nitrogen distribution in milk powders

Sample	% Casein and insoluble whey	% Soluble whey	% Lactalbumin and lacto-globulin
Raw skim milk	82.7	17.3	13.1
Spray-dried nonfat milk	83.7	16.3	11.7
Spray-dried nonfat milk, A	83.6	16.4	9.3
Instant dry milk No. 1, A	83.7	16.3	8.1
Spray-dried nonfat milk, B	84.6	15.4	9.3
Instant dry milk No. 1, B	84.7	15.3	7.9
Instant dry milk (demineralized aggregate)	86.7	13.3	6.5
Instant dry milk No. 2	86.5	13.5	6.6
Instant dry milk No. 3	86.3	13.7	8.0

the loss of amino nitrogen. Formol titrations to measure changes of amino nitrogen showed no difference between instant dry milk No. 1 and the spray dried nonfat milk from which it was made. Hence, wettability of instant dry milk cannot be ascribed to the formation of casein-lactose compounds.

A variety of dry milks were examined by the method of Rowland *(4)* to determine their protein nitrogen distribution. The data in Table 2 show that the main over-all protein change during the conversion of ordinary spray-dried nonfat milk to instant milk was a slight decrease in soluble lactalbumin and lactoglobulin fractions. This decrease was noted in both the milks which were readily wettable and those which were poorly wettable, and so, presumably, this decrease has no affect on dispersibility. Attempts to measure the changes in protein nitrogen distribution from the center of the instant milk particle toward the surface were unsuccessful.

Size and shape of the particles. A detailed microscopic examination of instant dry milk No. 1 showed that the individual particles consisted of large aggregates of smaller particles. Thus, in comparison with spray-dried nonfat milk which consist of 200-mesh size spherical particles, the instant milks are very large aggregates in the size range 80 to 20 mesh. This large difference in size suggested that it may be one of the prime factors affecting the dispersibility of instant dry milk. A correlation of the dispersibility and the particle size of a wide variety of dry milk shows (Table 3) that a general correlation exists between particle size and dispersibility. That the shape also affects the wettability of the dry milk is shown by the lower dispersibility time for spray-dried nonfat milk powder mixed with half its weight of celite. This mere separation of the powder particles by inert material reduced the dispersibility time from 180 seconds to the 40-50 second range.

In contrast to the rapidly wetting particles of instant dry milk the ordinary spray-dried nonfat milk particles are of smaller size and closer packed when they meet the water. Their smaller size has less effect on surface tension. As they begin to dissolve, some of the glass lactose dissolves readily and may form a saturated syrup at the water-powder interface, rendering solution of the remainder of the powder difficult. The hydrated lactose of the instant dry milk dissolves more slowly and this may reduce the tendency towards local saturated solutions.

TABLE 3

Dispersibility of milk powders

Sample	Mesh size	Gamma chloride ion per mm² of surface layer	Dispersibility time, seconds
Instant dry milk No. 1 (industrial preparation)	20-40	1.7	4.1
	20-40	2.1	4.0
	40-60	1.3	4.1
	60-80	0.8	4.9
	100	0.5	13-27
Instant dry milk No. 1 (experimental preparation)	20-40	1.6	4.2
	40-60	0.9	5.2
	60-80	0.6	7.5
Instant dry milk No. 2	20-40	1.4	4.4
	40-60	0.8	10.0
Instant dry milk No. 3	80	0.5	30-40
Spray-dried nonfat milk	~ 200	0.14	180
(with NaCl added before drying)	~ 200	0.3	180
(with KCl added before drying)	~ 200	0.3	180
(with NaCl + KCl added before drying)	~ 200	0.3	180
(with added Celite)	~ 200	0.14	38-48

Distribution of salts, lactose and protein. That the distribution of salts, lactose and protein in the individual particles may affect their wettability could be suggested from two observations. First, instant dry milk aggregates made by conversion of demineralized spray-dried nonfat milk wet very poorly as compared to the normal instant dry milk No. 1. This loss of wettability by the removal of ions suggests that they play a major role. Second, study of the conversion process for the preparation of instant dry milk No. 1 *(2, 3)* suggests that the critical stage in the conversion process might involve a reorientation of the components of dry milk because the more soluble components would be translocated to the surfaces of the particles as the slow drying proceeds. This hypothesis of a redistribution of components in the instant dry milk was tested by successive washings of dry milk particles and analysis of composition of the washings. The data obtained in this study are shown in Figure 2. As the percentage of instant dry milk washed off is decreased—that is, as the washings become more representative of the constituents at the outer surface—chloride, potassium and sodium ions increase, lactose increases, and calcium ion and protein decrease. Because of convenience of chloride ion analysis it was used as an indicator of the total ion concentration. The data in Figure 2 show that sodium and potassium ions are more concentrated at the outer surface of the instant dry milk than is calcium. The calcium ion concentration decreases toward the surface presumably because the calcium stays behind as the insoluble proteinate. Although the over-all composition of instant dry milk No. 1 is the same as the spray-dried nonfat milk from which it was prepared, the components of the spray-dried nonfat milk are equally distributed at the surface and at the interior of the particles as shown in the case of chloride ion and lactose in Figure 2. A study of the concentrations of sodium, potassium and calcium in the washings resulting when 22 and 30% of the spray dried nonfat milk were washed off, showed no differ-

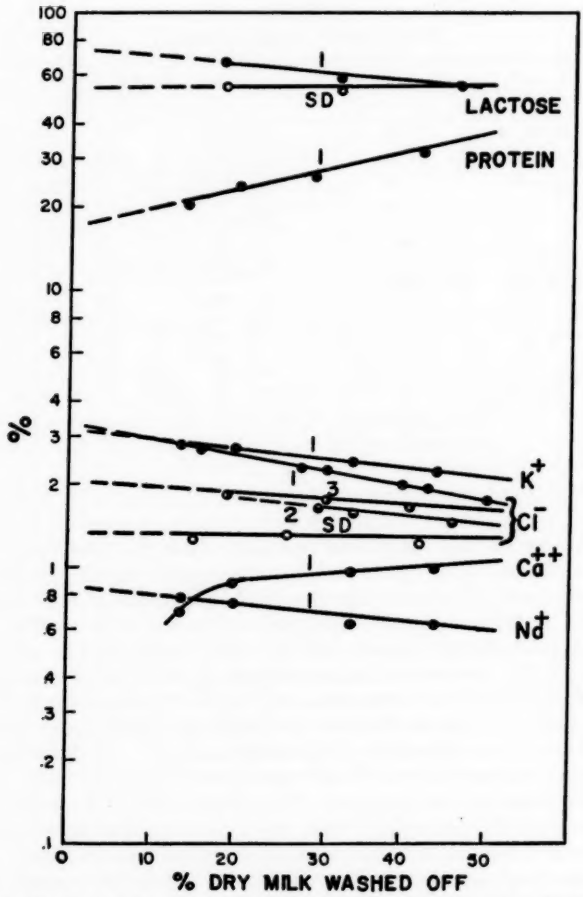

Figure 2. Changes in the amount of lactose, protein and ions as a function of the amount of dry milk washed off. The data for instant dry milks No. 1, 2, 3 and spray-dried nonfat dry milk are designated as 1, 2, 3 and SD respectively.

ence in ion concentration. Thus, we see a pronounced difference between ordinary spray-dried nonfat milk and instant milk made either by the conversion process or by spray-drying. Instant milk shows orientation of components, whereas ordinary spray-dried nonfat milk does not.

Further studies using chloride ion as the index of orientation showed the great importance of this orientation in increasing the wettability of milks. Extrapolation gives values of 2.0 to 3.4% chloride at the surface of instant dried milks as compared to 1.3% at the surface of ordinary spray-dried nonfat milk particles. The correlation between chloride concentration at the surface of the particles and the dispersibility time shown in Table 3 indicates the importance of orientation of components in de-

creasing dispersibility time. A mere increase of salt by addition of sodium and potassium chloride or their combination is not sufficient to decrease the dispersibility time of spray-dried milk.

CONCLUSIONS

From the evidence presented herein it may be concluded that the wettability of instant dried milk results mainly from the distribution of salts, lactose, and proteins, and the increased particle size. The large size and shape of the particles keep the individual particles farther apart as they reach the surface of the water. These heavier individual particles allow more rapid breaking of water surface tension. By these two effects each particle receives sufficient solvent for solution. The orientation of salt and lactose toward the surface provides readily soluble components for the rapid wettability. The less soluble components, calcium caseinate, for example, are oriented toward the center of the particles and are more readily dissolved after the salt and lactose have gone into solution.

SUMMARY

The state of the lactose and minor changes in protein apparently cannot account for the increased dispersibility of instant dry milks. Instant dry milks characteristically have a larger size and this aids dispersibility. As successive amounts of the instant dry milk particles are washed away, chloride, potassium, and sodium ions and lactose decrease indicating that they are preferentially oriented toward the surface. Calcium and protein concentrations increase showing preferential orientation in the interior. Spray-dried milk has uniform distribution of the components assayed—lactose and chloride. It is proposed that this orientation of readily soluble components on the surface of the instant dry milk aggregates greatly aids dispersibility.

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APPLICATION OF THE THIOBARBITURIC ACID TEST AS A QUANTITATIVE MEASURE OF DETERIORATION IN COOKED OYSTERS[a]

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Recent investigations *(7)* in this laboratory indicated that oysters which had been heated sufficiently to destroy the enzyme catalase underwent a different type of spoilage from that of raw oysters. This spoilage was characterized by the development of "stale" or "rancid fish" odors which were believed to be associated with unsaturated fat oxidation.

The TBA test has been applied to oysters in an attempt to establish a rapid objective method for determining oxidative rancidity. This test was selected for two reasons. First, it can be applied directly to the oyster tissue without the necessity of extracting the fat. Second, the fat decomposition product responsible for the test is obtained in much greater amounts from highly unsaturated fatty acids *(8)* such as are present in marine fats.

The test, based on a reaction described by Kohn and Liversedge *(9)*, has been shown by Bernhiem *et al. (1)* to measure oxidation products of unsaturated fatty acids. It depends upon the spectrophotometric estimation of a pink red compound produced when the acidified sample is heated in the presence of 2-thiobarbituric acid. The test has been applied to milk by Dunkley and Jennings *(5)* whose procedure has been investigated and modified for application to other dairy products by Biggs and Bryant *(2)*. A publication by Turner *et al. (14)* describes the application of the test to frozen pork.

MATERIALS AND METHODS

All oysters used were of the species *Crassostrea virginica*, tonged from Cat Point Reef in the Gulf of Mexico. They were purchased freshly shucked from a commercial packing house in Apalachicola, Florida.

A modification of the TBA test described by Turner *et al. (14)* for frozen pork has been adapted to oysters with minor adjustments. The detailed procedure follows:

One hundred g. of oysters are blended with 100 ml. of water in the Waring blender. Five g. of the oyster mixture, 5 ml. of 20% trichloroacetic acid in 2 M phosphoric acid and 10 ml. of 0.01 M 2-thiobarbituric acid are placed in a 50 ml. centrifuge tube. The tubes are then heated in boiling water for 10 minutes and chilled in an ice bath for 10 minutes. The pink colored TBA complex developed is extracted from the aqueous phase with 15 ml. of a solvent mixture consisting of 2:1 isoamyl-pyridine. Tubes are shaken vigorously for 2 minutes then centrifuged for 15 minutes at 2400 r.p.m. to break the emulsion formed during extraction. Five ml. of the solvent extract is then diluted with 5 ml. of the solvent. A portion of the mixture is placed in a cuvette and the color is measured at 540 mμ against a solvent blank in a Beckman spectrophotometer.

[a] The research reported in this paper was conducted by Florida State University under a contract with the U. S. Fish and Wildlife Service. It was financed with funds made available under provisions of Public Law 466, 83rd Congress, approved July 1, 1954, generally termed the Saltonstall-Kennedy Act. The study was made possible through the cooperation of the Oceanographic Institute, Florida State University.

RESULTS AND DISCUSSION

When the TBA reagent is applied to either raw or cooked tissue, some pink color is obtained even with fresh oysters. The extracts all show sharply defined absorption peaks with maxima at 540 mμ (Figure 1), characteristic of the TBA colored complex. However, an interfering yellowish brown pigment similar to that described by Caldwell *et al. (3)* in cereal products, was found in the oyster extracts. These workers removed the pigment by selective absorption. Interference from other colored compounds extracted from the whole food may also be eliminated by performing the test on steam distillates of the material to be analyzed *(12, 13)*.

Since either of the above procedures entails an additional, rather tedious step in the TBA analysis, it was decided to investigate other methods of removing the pigment and to evaluate the degree of interference if the pigment were not removed.

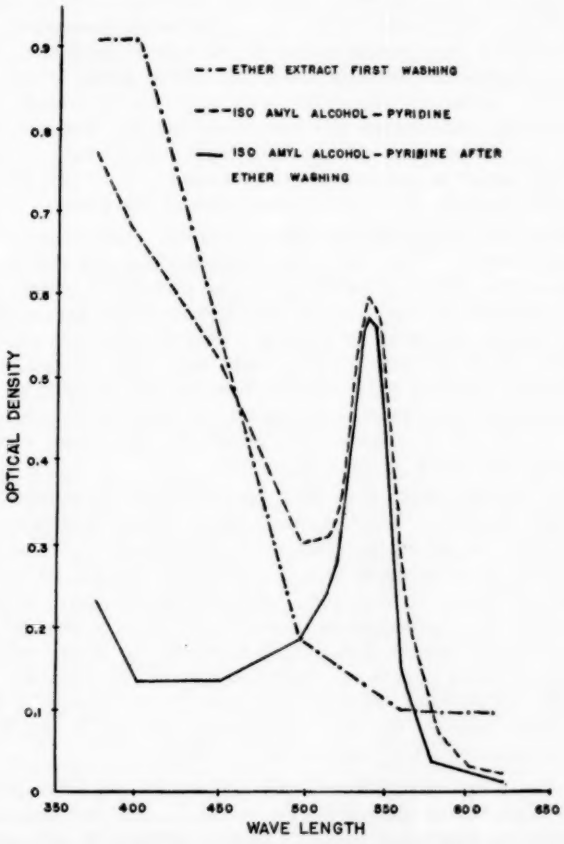

Figure 1. Absorption curves of oyster extracts with and without ether washings.

To determine if the interfering pigment could be removed without simultaneous extraction of the pink complex, oyster tissues, after reaction with the TBA reagent, were extracted with a number of different solvents (isoamyl alcohol, pyridine, 2:1 mixture of isoamyl alcohol and pyridine, chloroform, ethyl ether) and the absorption at various wave lengths read with each solvent. Of the solvents used, ethyl ether gave the most complete extraction of the yellow interfering pigment without removing any of the pink TBA complex. Three washings with 15 ml. portions of ethyl ether were found necessary to remove all of the yellow color. The other solvents tested also removed the yellow color but with varying amounts of the pink colored complex. The 2:1 mixture of isoamyl alcohol and pyridine gave most complete extraction of the pink color.

Figure 1 shows absorption curves obtained from an ether extract of oyster tissue and from isoamyl alcohol-pyridine (2:1) extracts of aliquots of the same oyster tissue, with and without preliminary ether washings. It will be observed that the ether washings show no peak in the 540 mμ region of TBA complex absorption, but progressive absorption at lower wave lengths which may be attributed to the yellow interfering pigment. Isoamyl alcohol-pyridine extracts made on oyster tissue which had not been previously extracted with ether were a yellowish orange color and showed considerable absorption at lower wave lengths. However, the reading obtained at 540 mμ was only slightly higher than that obtained after ether washings, which would indicate that, except at very low TBA values, the interfering pigment does not seriously distort the results.

Changes in TBA value with storage of oysters. To determine the correlation, if any, between TBA values and organoleptic changes in raw and cooked oysters, the following experiment was performed. Fresh uncooked and cooked oysters from the same lot were stored for 12 days at 4° C. The cooked oysters were prepared by placing 1 pint of oysters in an aluminum frying basket, allowing them to drain, and then immersing them in 1½ quarts of boiling distilled water and holding for 2½ minutes at 90-95° C. Catalase was inactivated by this treatment. A number of 100 g. samples of both raw and cooked oysters were placed in glass jars, covered with aluminum foil and stored in the refrigerator.

The TBA test was used with both raw and cooked samples with and without ether washing. The test was run at 3-day intervals and odor comparisons were made at the same time. Both raw and cooked oysters were rated fresh in odor up to and including the 6th day of storage. On the 9th day a definite "rancid fish" off-odor was apparent in the cooked sample, and this odor increased in intensity between the 9th and 12th day of storage. Figure 2 shows that the TBA values for these cooked oysters changed sharply after 6 days and increased rapidly through the 12th day. Thus, in this and another similar experiment there appears to be a very close correlation between the time of a sharp increase in the TBA value and the development of the "rancid fish" off-odor.

In this and other experiments, raw oysters do not show a consistent increase in TBA values during storage at 4° C., but fluctuate erratically until bacterial spoilage is evident. The raw oysters do not develop the typical "rancid fish" odor *(7)*.

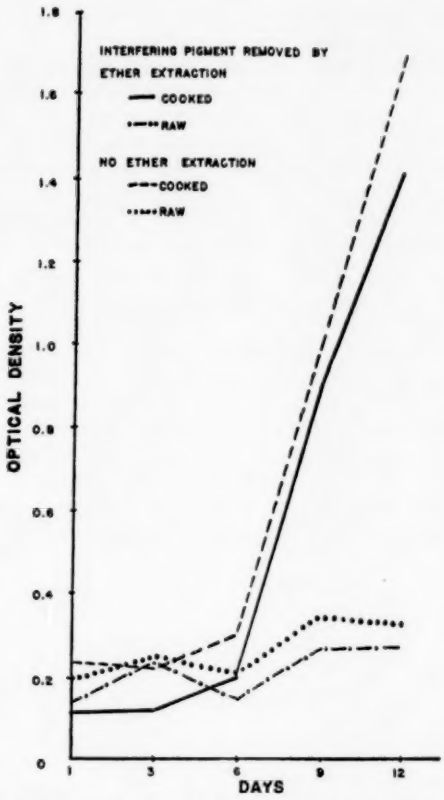

Figure 2. The effect of storage on TBA values of raw and cooked oysters.

Insufficient data are available to establish a TBA value at which cooked oysters may be declared rancid. There is a definite induction period in the oxidation of the fat during which the TBA value does not increase significantly over the values obtained on fresh oysters. This induction period, characteristic of natural fats, is presumably due to the antioxidant effect of tocopherol present in the fat. However, at the end of this induction period, the TBA value increases so rapidly that it would appear to be an extremely sensitive measure of deterioration in cooked oysters.

A comparison of the curves obtained on the same material with and without ether extraction shows that the interfering pigment is fairly constant in amount at all storage periods. Its concentration in the extract (i.e., the difference between the two curves) corresponds to an average optical density of 0.10. This represents about half of the total absorption of fresh oysters, but becomes only a small fraction of the total absorption in rancid samples. In the use of this method as a rapid practical test for the evaluation of cooked oysters, we believe that the ether washing may

be omitted and the TBA value of the fresh oysters set at a correspondingly higher value, i.e., .20 to .30.

Addition of antioxidant to cooking water. Several primary fat antioxidants have been used with success in preventing or retarding rancidity in various fats and foods with a high fat content. Among these are butylated hydroxyanisole (BHA) and nordihydroguairaretic acid (NDGA), phenolic compounds which have been reported as effective antioxidants for lard and other animal fats *(4, 10, 11)*. Oxidative changes in frozen shrimp have also been inhibited by BHA *(6)*. Using a commercial mixture, Tenox N, Gardner and Watts *(7)* were able to inhibit the oxidative change characterized by a "rancid fish" odor in cooked oysters.

Fresh oysters were cooked for 2½ minutes at 90-95° C. in the manner previously described, proportions used being one pint of oysters (drained) to 1½ quarts of water. A commercial mixture, Tenox N, containing 74% propylene glycol, 20% BHA, 4% anhydrous citric acid and 2% NDGA was added to the water prior to boiling in sufficient quantity to make the final concentration of the antioxidant in the water 0.1% BHA. As controls, oysters were cooked in the above mentioned proportions without the addition of the antioxidant. A number of 100 g. samples of both cooked oysters without antioxidant and cooked oysters with antioxidant were placed in glass jars, covered with aluminum foil and stored in the refrigerator at 4° C.

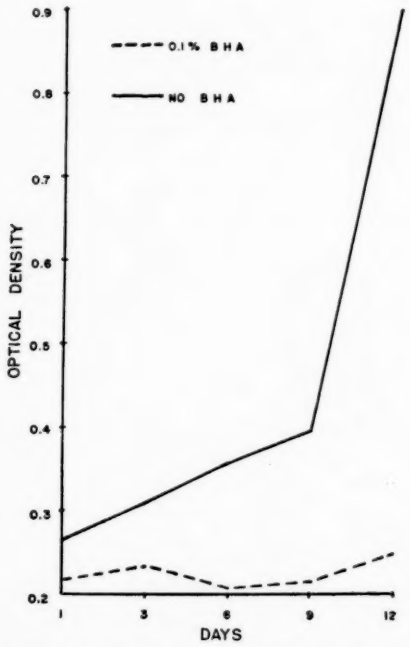

Figure 3. The effect of 0.1% BHA on the TBA value of cooked oysters.

The TBA test was conducted on both cooked oysters without antioxidant and cooked oysters with antioxidant. The test was run at 3-day intervals, over a period of 12 days, and odor comparisons were made at the same time.

The same general pattern that had been established by the cooked oysters in the previous experiment was repeated. On the 9th day a definite "rancid fish" off-odor with an increase in intensity between the 9th and 12th day of storage was apparent in the cooked sample without antioxidant. Samples of cooked oysters with the antioxidant retained the "oyster" odor. The TBA values in both samples correlated well with the organoleptic comparisons (Figure 3).

It seems clear that the off-odors occurring in cooked oysters involve unsaturated fat oxidation and may be inhibited by the addition of antioxidants. The significance of these results can be better appreciated when we think of cooked oysters as the basis for such commercial products as oyster stew, oyster sticks, fried oysters, etc. At present a series of antioxidants and combinations of antioxidants are being used to observe their effect on cooked oysters that have been stored in a freezer. The TBA test is being applied in conjunction with this experiment.

SUMMARY

A simple modification of the 2-thiobarbituric acid test for oxidative rancidity has been adapted to oysters. The test may be performed directly on oyster tissue without previous extraction of the fat.

As measured by this test, refrigerated cooked oysters have a definite induction period during which the TBA values do not increase over those for freshly cooked samples. At the end of the induction period there is a very rapid increase in the TBA values which corresponds closely with the development of "rancid fish" odors. Uncooked refrigerated oysters do not show consistent increases in TBA values nor do they develop rancid odors.

The "rancid fish" odor, and elevated TBA values typical of oysters cooked enough to inactivate catalase, have been retarded by the addition of an antioxidant preparation.

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QUALITY EVALUATION AND CHEMICAL COMPOSITION OF SOY SAUCE

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Soy sauce is one of the important condiments used by Oriental peoples. Its quality varies greatly with the raw materials used and methods of manufacture (by fermentation or by acid-hydrolysis). In the fermentation process *(13, 18)*, cooked soya bean and wheat flour are fermented by the mold *Aspergillus oryzae* and then by yeast; this is followed by long digestion in brine *(12)*. In the hydrolysis process *(5)*, defatted soya bean cake is heated with inorganic acid under reflux or under pressure and then neutralized with sodium carbonate or sodium hydroxide. In both processes, polypeptide bonds in protein are hydrolyzed into peptides and amino acids. Generally, the product of fermentation has more aromatic and pleasing flavor; however, it is more costly. The acid-hydrolyzed product may have some undesirable flavor and aroma because of the formation of degradation products during hydrolysis. It may also lack aromatic and flavor compounds which are formed only in the fermentation process.

Literature on soy sauce is mostly in Oriental languages, and information is lacking on the relationship between flavor acceptance and chemical composition of soy sauce. In this report, seven soy sauce samples obtained from markets in California were compared for flavor and chemical composition. The importance of salt, acidity, amino acids, and total nitrogen to flavor acceptance is discussed.

EXPERIMENTAL MATERIALS AND METHODS

Soy sauce. Samples of 7 brands of soy sauce obtained from markets in California were used. They were all packed in glass bottles and contained 0.1% sodium benzoate.

Flavor and aroma. The flavor and aroma of the samples were evaluated organoleptically by a panel of 4 persons. A randomized incomplete block design was used. The samples were served 3 at a time on 14 separate days in such a manner that each sample was served 6 times, and each sample was served along with each other sample twice. At each session, the judges were asked to rank the samples in the order of their preference for aroma and flavor. Samples were scored as follows: 1, *good;* 2, *intermediate;* and 3, *poor.* The results were analyzed statistically for least significant difference (L.S.D.), and probability level of significance.

Color. Two ml. of the sample was diluted with distilled water to 200 ml. in a volumetric flask. The color of the diluted sample was measured in a Klett-Summerson photoelectric colorimeter, using a blue filter.

Specific gravity. Specific gravity was determined with a 25-ml. pycnometer.

Total solids. Total solids were determined by drying 10 ml. of the sample in porcelain dishes at 70° C. under 27 in. of vacuum for 12 hours.

Total titratable acidity. Five ml. of the sample was mixed with 150 ml. of distilled water and titrated with standard 0.1 N sodium hydroxide to pH 8.0, using a Beckman pH meter. The results were expressed as milliequivalent of acid per 100 ml. The same solution was used for analysis of amino nitrogen content.

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[a] Present address: Hershel California Fruit Products Co., Inc., Woodland, California.

Sodium chloride. Salt content in the sample was determined by the $AgNO_3$-KCNS titration method *(4)*.

Total and amino nitrogen. The improved Kjeldahl method *(4)* was used to determine total nitrogen content. Amino nitrogen was determined by the Sorensen formol-titration method *(4)* modified as follows: The sample from total acid determination was treated with 5 ml. of 40% formaldehyde solution and titrated with 0.1 N sodium hydroxide to pH 8.0. A blank was run using 5 ml. of distilled water instead of the sample. Results were reported as mg. amino nitrogen per 100 ml. sample.

Paper chromatography of amino acids. Ten ml. of the sample was passed through a Dowex 50 × 4 cation exchange resin column (3.5 cm. in diameter and 37 cm. high) in the hydrogen form. The column was washed with 100 ml. of distilled water to remove chloride and other anions. Amino acids were eluted from the column with 60 ml. of 2 N NH_4OH to get an alkaline effluent. Eluate was evaporated under vacuum to about 5 ml., and passed an Amberlite IRA-400 anion exchange column in the hydroxyl form. The column was washed with 100 ml. of distilled water. Amino acids were eluted from the column with 60 ml. of 2 N HCl to obtain an acid reaction in the eluate. Eluate was evaporated under vacuum to 10 ml. A 0.05-ml. aliquot was chromatographed on a 22″ × 18″ Whatman No. 1 filter paper, first with phenol:water (80:20) for 40 hours at 70° F. A beaker containing 100 mg. of sodium cyanide and 6 ml. of water was placed in the chamber to retard decomposition of phenol. The paper was dried under a ventilated hood for 24 hours, then chromatographed in the second dimension with butanol: acetic acid:water (4:1:5) for 20 hours at 70° F. The paper was dried under ventilation, sprayed with 0.2% ninhydrin in 95% ethyl alcohol, dried, and then heated at 180° F. for 10 minutes. The amino acids were identified by comparing their R_f values with those of known amino acids *(6)*.

RESULTS

Flavor, aroma, and color. Flavor and aroma scores and the color ratings of the 7 soy sauce samples are shown in Table 1. The samples can be divided into 3 grades with respect to aroma and flavor. Samples 1, 2, and 3 were ranked as best in both aroma and flavor. Among these 3 samples there was no significant difference at a probability level of 5%. Samples 4 and 5 are of intermediate quality; samples 6 and 7 were graded as poor. A great difference in optical density was found among samples when they were diluted 1:100 with distilled water before being measured in the photoelectric colorimeter. No correlation exists between color and aroma and flavor of the samples. This might be explained by the fact that the color of soy sauce is generally derived from caramel, added during the manufacturing process. Unless poor-quality caramel is used for coloring, aroma and flavor are not related to color. As soy sauce contributes not only to flavor and aroma but also to color of the cooked foods, use of the photoelectric method described above is desirable as an aid in controlling quality.

TABLE 1

Flavor, aroma and color of soy sauce[1]

Sample No.	Average aroma rank (1 = best 3 = worst)	Average flavor rank (1 = best 3 = worst)	Color as optical density[2] (diluted 100 times)
1	1.29	1.33	0.337
2	1.25	1.33	0.467
3	1.58	1.46	0.409
4	2.12	2.17	0.636
5	2.29	2.33	0.341
6	2.71	2.54	0.542
7	2.75	2.84	0.644

L.S.D. at 1% level: 0.35.
L.S.D. at 5% level: 0.28.

[1] All samples within blocks ranked 3 at a time in a 14-incomplete block design for flavor and aroma by 4 experienced judges.

[2] Calculated from Klett Summerson photoelectric colorimeter readings, using a blue filter.

TABLE 2

Physical and chemical properties of soy sauce

Sample No.	Specific gravity	Total solids %	pH	Total acid m.e./ 100 ml.	Sodium chloride g./100 ml.	Total nitrogen g./100 ml.	Amino nitrogen	
							g./100 ml.	% of total nitrogen
1	1.1841	31.17	4.80	16.15	18.7	1.40	0.44	31.4
2	1.1836	32.62	4.57	19.97	19.1	1.35	0.44	32.6
3	1.1699	28.68	4.81	10.64	20.4	1.25	0.51	40.8
4	1.1323	25.12	4.80	6.13	13.4	1.09	0.52	47.7
5	1.2228	38.58	5.00	7.36	20.2	1.18	0.54	45.7
6	1.1956	34.43	4.70	10.52	19.2	1.05	0.38	36.2
7	1.2698	45.17	4.52	10.22	27.2	0.50	0.13	26.0

Physical and chemical properties. Physical and chemical properties of the samples listed in Table 1 are shown in Table 2. Samples with high specific gravity were also high in total solids content, both of which contribute to the body of soy sauce. Salt content varied between 13.4 and 27.2 g./100 ml. Samples 1, 2, and 3, which were ranked best in aroma and flavor, have a sodium chloride concentration ranging between 18.7 and 20.4 g./100 ml. It is important to point out that salt concentration is only one of the factors influencing the flavor. Salt content of samples 5 and 6 was in the optimum range, but they were ranked low in flavor.

The pH values of the samples varied from 4.52 to 5.00, but the difference in pH values cannot be used to explain their difference in flavor. This is obvious when the pH values of samples 1 and 4, and 2 and 7, in Table 2, are compared with the flavor score of the same samples in Table 1. Total titratable acidity varied between 6.13 to 19.97 m.e./100 ml. Samples 1 and 2, which were rated best in flavor and aroma, had a higher total acidity than the rest of the samples.

Total nitrogen and amino nitrogen contents bear some relationship to the flavor score of soy sauce. Samples 1, 2, and 3, which were scored best in flavor, contained higher total nitrogen than the other samples. The percentage of amino nitrogen in total nitrogen, and also the quantity of amino and total nitrogen, are important factors influencing quality. In samples 1 and 2, amino nitrogen constituted only 31.4 and 32.6% respectively of the total nitrogen, whereas it amounted to 47.7 and 45.7% in samples 4 and 5, which were made by the acid-hydrolysis process. Samples 6 and 7 are low in both total and amino nitrogen, which might account for their poor flavor score even though the amino nitrogen percentages are lower than those of samples 4 and 5.

Amino acids. A two-dimensional chromatogram of the amino acids of sample 1 (Table 1) is shown in Fig. 1. The following amino acids were found: alanine, arginine, aspartic, glutamic, leucine, lysine, phenyl alanine, proline, serine, threonine, and valine. Of these, glutamic, alanine, leucine, and aspartic acid are present in larger quantities than the others. The same amino acids were found in sample 4 (Table 1), made by the acid-hydrolysis process, which also contained small quantities of methionine and glycine. Cystine, histidine, and tyrosine were not found in either sample.

DISCUSSION

The difference in flavor and aroma of the soy sauce samples might be explained in part by their difference in chemical composition. The optimum concentration of salt, which is important to flavor acceptance, seems to lie between 18.7 and 20.4 g./100 ml. An unusually high concentration definitely impairs the flavor, as is shown by the flavor score and salt concentration of sample 7 in Tables 1 and 2. A low concentration of sodium chloride (13.4 g./100 ml. of sample 4, Table 2) may create a problem of mold growth during storage. Thus, salt concentration is also important to keeping quality. Total acidity also appears to be important to flavor

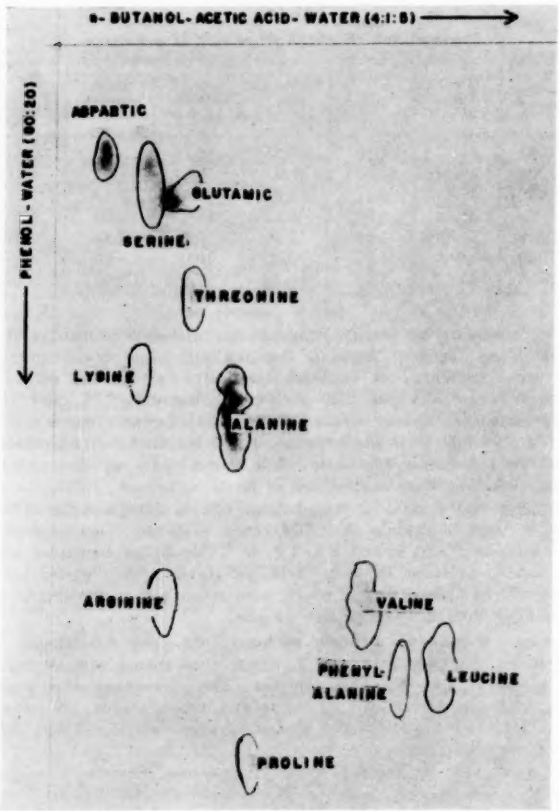

Figure 1. Two-dimensional chromatogram of amino acids in salt-free soy sauce. Phenol:water (4:1 v/v), butanol:acetic acid:water (4:1:5 v/v).

acceptance. Samples 1 and 2, which were higher in total acidity, were ranked higher in flavor. The acids may be metabolic products formed during fermentation, or they may be derived from some of the amino acids. A high percentage of amino nitrogen is an indication of the acid-hydrolysis process. This is another way of differentiating a soy sauce made by hydrolysis from those made by fermentation. Ichikawa *(8)* found maltose in fermented soy sauce but not in the acid-hydrolyzed product. This finding can also be used to differentiate the two products.

There is a slight difference between the amino acid content reported herein and the data in the literature *(9, 15, 17)*. For instance, serine has not been reported in other analyses; however, it was found in the samples used in this investigation. Furthermore, cystine, histidine, and tyrosine, which were found in the samples analyzed by Yoshino *(17)*, were not found in samples investigated here. Apparently, either the samples were different, or different analytic methods may account for the discrepancy.

As the presence of large amounts of sodium chloride in soy sauce tends to block the movement of amino acids on the paper chromatogram, the salt must be removed before chromatography. The method for separating amino acids from salt in soy sauce involves ether extraction and precipitation of the amino acids as mercuric salts *(15, 17)*, a rather tedious and time-consuming process. Use of ion-exchange resin to remove salt, as described in this report, was a quicker and simpler way of achieving the same purpose; this method might also be used to identify amino acids in other foods high in inorganic salts.

The flavor of soy sauce is related to the amount of salt, amino acids, organic acids, sugars *(8, 16)*, and other flavoring constituents. Data presented in Table 1 and 2 explain in part the differences in flavor score. The aroma of soy sauce may be due to the volatile aromatic compounds. Possible contributors to the aroma of soy sauce are: (a) aldehydes: isovaleraldehyde *(1)*, beta-methyl-mercapto propionaldehyde *(3)*, and propionaldehyde and butyraldehyde *(13)*; (b) alcohols: 3-methyl-mercapto propyl alcohol *(2, 11)* and ethyl alcohol *(10)*; and (c) esters: isoamyl lactate *(10)* and ethyl acetate *(1, 10)* and an acetal *(14)*. More work is needed to determine the quantity, formation, and relative merit of these constituents.

In conclusion, the quality of soy sauce is still not well understood. This report serves only to initiate study and to stress the importance of chemical constituents to flavor. Information is needed on quantitative analyses of amino acids and the relative merit of each to flavor and nutrition. A chemical test for volatile compounds, organic acids, sugars, and other constituents which might be related to the acceptance of soy sauce is equally important.

SUMMARY

Soy sauce samples obtained from markets in California were evaluated for flavor, aroma, and color. The organoleptic results are compared with the chemical composition of the samples. Significant differences in flavor and aroma were found. Salt, acidity, and nitrogen content are important factors influencing flavor acceptance. Amino acids in soy sauce, as identified by two-dimensional paper chromatography, were found to be alanine, arginine, aspartic acid, glutamic acid, glycine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, and valine. Of these, glutamic acid, alanine, leucine, and aspartic acid are present in larger quantities than the others. A rapid ion-exchange method to remove salt from soy sauce for paper chromatography study is described. The importance of various chemical constituents to flavor is discussed.

Acknowledgment

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EFFECTS OF IONIZING RADIATIONS ON PLANT TISSUES. II. SOFTENING OF DIFFERENT VARIETIES OF APPLES AND CARROTS BY GAMMA RAYS[a, b]

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In a previous paper *(2)* an objective method was described for evaluating the softening effect of gamma radiation on one variety each of apples, beets, and carrots. These three types of plant tissues showed significantly different threshold dosage values[e] but a similar radiation dosage-softening relationship. The latter was indicated by the similar angles of the straight lines obtained when the logarithm of dosage was plotted against softening as expressed by the percentage decrease in crushing loads.

The present study was undertaken to establish whether the differences in the threshold dosages of apples and carrots were real rather than an incidental result of the particular variety or maturity chosen. Therefore the irradiation-induced softening was studied on seven varieties of apples and five varieties of carrots. Further, it was desirable to establish whether the linear log dosage-effect relationship held in the case of different varieties, since comparisons were previously made on the basis of threshold dosages derived from this linear relationship.

EXPERIMENTAL MATERIALS AND METHODS

The seven varieties of apples selected for these tests were different in respect to parentage, commercial usage, date of harvest, storage life, etc. The apples were grown in the Experiment Station orchards, picked at a maturity most commonly used for commercial storage, then stored at 31° F. without special provision for purifying the atmosphere. The characteristics of the selected varieties *(6)* are given in Table 1. The 5 varieties of carrots were obtained from Experiment Station variety plots near Greece, N. Y., and represent several important commercial varieties. They were harvested in early November, 1955, and kept in common storage until used.[f]

Methods of preparing the samples for irradiation, procedure used in the irradiation with gamma rays from a Co^{60} source, and measurement of tissue firmness have been

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[a] Approved by the Director of the New York State Agricultural Experiment Station, Geneva, as Journal Paper No. 1048, September 11, 1956.

[b] This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, and has been assigned No. 691 in the series of papers approved for publication. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the Department of Defense.

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[e] The threshold dosage (TD) is the minimum amount of radiation which causes a detectable degree of softening *(1, 2)*.

[f] We are indebted to Mr. Leo Klein of our Pomology Department and Dr. John Atkin of our Vegetable Crops Department for their help in selecting and supplying the apples and carrots, respectively.

TABLE 1

Characteristics of the seven apple varieties used in irradiation tests[1]

Variety	Suitability for						
	Dessert	Freezing	Dehydration	Sauce	Juice	Canning	Baking
Baldwin	P	E	E	E-G	G	G	G
R. I. Greening	P	E	G	E	G	G	F
Monroe	G	E	E-G	E	G	G	G
Rome	F	G-F	G	G	G-F	G	E
Gravenstein	G	G	....	G	F	....	P
McIntosh	E	G	....	F-P	G-F	F	P
Golden Delicious	E	G	F	P	F-P	F	P

[1] The original data have been rearranged to show trends in their desirability for various purposes, expressed by the symbols E = excellent, G = good, F = fair and P = poor. The ratings for Monroe apples were made by Mr. Leo Klein of the Experiment Station staff. The ratings for all other varieties were taken from Table 7, in "Apples and Apple Products" by Smock and Neubert, (ref. 6).

described previously *(2)*. Briefly, tissue cylinders 18 mm. in diameter and 10 mm. high were wrapped in Saran film and irradiated at the University of Rochester.[5] Different irradiation dosages were obtained by placing the cylinders at various distances from the Co^{60}. The irradiation usually took 18 hours. Afterwards the samples were brought to Geneva and the firmness of the tissues was measured by an instrument which records the load required to crush the cylinders *(2, 5)*.

Crushing loads were measured on single cylinders of carrot tissues. However, 4, 6, or 8 cylinders of apple tissue had to be used in order to give sufficiently high readings for the unirradiated controls. Since there is no exact proportionality between the number of cylinders used and the crushing load per cylinder of the same raw material, the same number of cylinders was used throughout each group of measurements. In accordance with previously reported requirements *(2)* for a satisfactorily low standard error of the mean (SEM), at least 5 and sometimes as many as 10 replicate firmness measurements were performed on groups of cylinders irradiated at the same dosage. The means of these crushing loads are given in Table 2 for apples and Table 4 for carrots.

The method of linear regression *(7)* was used to calculate the constants for the best straight lines fitting the points derived from each experiment. These constants were then used to obtain the angles of the lines and the threshold dosage (TD) values. In addition the dosages required to effect 50% change in the crushing load of the tissues (CD_{50}) were also computed. These values are listed in Table 3 for apples and in Table 5 for carrots. Constants were also calculated for the best line passing through the points for all varieties of apples and of carrots and these average lines are shown in Figure 1.

RESULTS AND DISCUSSION

Measurements presented in Table 2 are for the group of apple tissue cylinders used in any given experiment. The number of cylinders varied from 4 to 8. Crushing load per cylinder in the unirradiated (control) apples ranged from 9.0 to 26.0 lbs. Gravenstein and Baldwin apples were the firmest with 20.5 and 26.0 lbs. per cylinder crushing loads, respectively; all the other samples had initial firmness in the range of 9.0-15.5 lbs. crushing load per cylinder. Of course, firmness readings before irradiation also depended on the dates when these tests were made, and the three varieties of apples which were tested repeatedly showed progressive softening in storage.

[5] We are indebted to Dr. L. W. Tuttle for his kind cooperation.

TABLE 2

Softening effect of gamma rays on seven varieties of apples

Variety and date of irradiation	Dosage $r \times 10^{-3}$	Crushing[1] load, lbs.	Crushing load per cylinder, lbs.	± SEM lbs.	Variety and date of irradiation	Dosage $r \times 10^{-3}$	Crushing[1] load lbs.	Crushing load per cylinder, lbs.	± SEM lbs.
Gravenstein	None	82	20.5[2]	1.6	Monroe	None	80	13.3	2.1
9/14/55	19.8	83		1.6	12/15/55	32.3	76		1.5
	51.1	78		2.0		58.6	75		2.6
(4 cylinders)	55.2	70		2.4	(6 cylinders)	102.0	72		2.6
	96.0	60		1.6		211.0	67		5.6
	198.0	42		0.9		544.0	37		2.4
Golden	None	97	12.1	5.6	Monroe	None	71	11.6	1.3
Delcious	16.2	97		2.7	12/22/55	113.0	62		1.3
12/8/55	54.1	91		1.8		233.0	55		1.9
	88.5	83		4.2	(6 cylinders)	449.0	47		0.9
(8 cylinders)	194.0	70		2.8		601.0	34		1.7
	502.0	43		2.3					
R. I. Greening	None	67	11.2	2.4	Monroe	None	56	9.3	2.6
12/15/55	32.3	66		2.9	2/8/56	99.9	46		2.7
	58.6	61		3.6		206.0	41		2.4
(6 cylinders)	102.0	58		5.7	(6 cylinders)	398.0	35		1.9
	211.0	53		3.9		534.0	28		2.6
	544.0	33		2.6					
McIntosh	None	72	9.0	1.8	Baldwin	None	104	26.0	3.4
12/28/55	61.2	59		2.3	12/8/55	16.2	98		2.6
	106.0	56		2.1		54.1	85		3.4
(8 cylinders)	220.0	44		1.2	(4 cylinders)	88.5	78		3.1
	568.0	26		2.1		194.0	50		2.4
	2,210.0	0		....		502.0	34		1.6
Rome	None	86	10.8	1.3	Baldwin	None	62	15.5	2.7
12/28/55	106.0	63		0.4	1/19/56	117.0	32		2.0
	220.0	56		3.5		241.0	23		1.5
(8 cylinders)	568.0	38		3.2	(4 cylinders)	465.0	22		2.8
	2,210.0	0		....		622.0	14		1.7
Rome	None	73	9.1	4.6	Baldwin	None	48	12.0	2.0
1/19/56	117.0	59		4.9	2/8/56	99.9	43		3.4
	241.0	44		2.9		206.0	27		1.1
(8 cylinders)	465.0	36		1.2	(4 cylinders)	398.0	19		2.3
	622.0	30		6.8		534.0	19		2.5

[1] These results are the means of total crushing loads required for the number of cylinders shown in parentheses. Five or more replicate determinations were made on groups of cylinders irradiated at each dosage, in order to reduce the standard error of the mean (SEM) to a satisfactorily low level.

[2] These values are given to allow a comparison of the firmness of the apples used in the tests.

Characteristics of the apple varieties used in these tests, as described in Table 1, do not seem to bear any relation to their response to irradiation as indicated by the TD and CD_{50} values listed in Table 3. In fact, the extent of radiation-induced softening varied considerably with apples of the same variety tested at different times and the ranges of TD and CD_{50} values within varieties differ as much as the varietal averages do.

TABLE 3

Constants of lines representing log irradiation dosage vs. per cent change in crushing load for 12 lots of apples

Variety	Date[1]	Angle	TD $r \times 10^{-3}$	CD_{50} $r \times 10^{-3}$
Gravenstein	9/14/55	89° 3′	31.2	212
Golden Delicious	12/ 8/55	88° 59′	53.8	419
R. I. Greening	12/15/55	88° 55′	66.6	579
McIntosh	12/28/55	88° 50′	31.7	330
Rome	12/28/55	88° 34′	30.0	537
Rome	1/19/56	88° 54′	46.4	428
Monroe	12/15/55	89° 18′	107.0	437
Monroe	12/22/55	88° 40′	57.3	834
Monroe	2/ 8/56	88° 34′	38.1	687
Baldwin	12/ 8/55	88° 56′	24.9	220
Baldwin	1/19/56	88° 19′	4.2	123
Baldwin	2/ 8/56	89° 4′	50.5	325
All varieties[2]		88° 46′	34.3	400

[1] Date of irradiation.
[2] Values for line obtained by linear regression to represent 43 points from all experiments on all varieties listed in Table 2. As noted, values below 10% change and showing 100% change in crushing load were not used in any of the computations.

TABLE 4

Softening effect of gamma rays on five varieties of carrots

Variety and date of irradiation	Dosage $r \times 10^{-3}$	Crushing[1] load, lbs.	± SEM lbs.	Variety and date of irradiation	Dosage $r \times 10^{-3}$	Crushing load lbs.	± SEM lbs.
Chantenay	None	92	1.2	Red Core	None	94	8.6
10/13/55	601.0	59	2.7	Chantenay	100.0	98	12.2
	1,278.0	29	1.8	2/8/56	206.0	89	8.1
	3,670.0	4	1.7		398.0	72	6.3
					534.0	70	5.6
Royal	None	93	7.9	Long	2,070.0	32	2.1
Chantenay	111.0	108	11.7	Imperator			
1/27/56	230.0	85	2.9	1/27/56	None	124	3.1
	444.0	60	5.8		111.0	105	1.5
	595.0	45	2.9		230.0	83	2.8
					444.0	93	6.8
Danvers	None	107	6.7	Long	595.0	73	3.9
1/27/56	111.0	106	10.3	Imperator			
	230.0	103	10.5	2/8/56	None	109	4.3
	444.0	74	4.3		100.0	105	4.7
	595.0	77	3.3		206.0	92	4.1
					398.0	80	5.7
Red Core	None	93	4.5		534.0	72	6.8
Chantenay	111.0	92	4.3		2,070.0	31	4.5
1/27/56	230.0	86	3.5				
	444.0	74	2.8				
	595.0	70	6.2				

[1] These results are the means of crushing loads required for one cylinder. Eight or more replicate determinations were made on cylinders irradiated at each dosage.

TABLE 5

Constants of lines representing log irradiation dosage vs. per cent change in crushing load for seven lots of carrots

Variety	Date[1]	Angle	TD $r \times 10^{-3}$	CD_{50} $r \times 10^{-3}$
Chantenay	10/13/55	89° 14′	178.0	842
Royal Chantenay	1/27/56	89° 11′	132.0	671
Danvers	1/27/56	88° 43′	126.0	1,680
Red Core Chantenay	1/27/56	88° 39′	152.0	2,310
Red Core Chantenay	2/ 8/56	88° 53′	135.0	1,250
Long Imperator	1/27/56	87° 50′	23.5	1,860
Long Imperator	2/ 8/56	89° 8′	137.0	774
All varieties[2]		88° 57′	117.0	982

[1] Date of irradiation.
[2] Values for line obtained by linear regression to represent 29 points from all experiments on all varieties as listed in Table 4.

Points obtained in the various experiments and the average line representing the radiation-induced softening of all apple samples are shown in Figure 1. In all experiments the points fell on or near the calculated lines indicating a straight-line relationship between log radiation dosage and per cent change in the firmness. The TD and CD_{50} values of these lines, together with the calculated angles that they make with the abscissa are given in Table 3. The angles for the lines for the various experiments on apples are all within the range 88° 19′-89° 18′, indicating that the dosage vs. effect relationship is essentially constant. All except a few of the lines are quite similar in their CD_{50} values. The TD values differ more.

Carrot data in Tables 4 and 5 and the average line representing all data in Figure 1 show the results obtained on different varieties. Single cylinders were used in the measurement of the crushing loads of carrot tissue. Considerable variation could be observed between successive experiments with carrots of the same variety and the constants in Table 5 indicate variation in TD and CD_{50} values and the angles formed with abscissa. Angles for the lines for the various carrot experiments range from 87° 50′ to 89° 14′. The average line for carrots (Figure 1) with an angle of 88° 57′ is nearly parallel with the average line for apples (88° 46′).

Thus, when expressing dosage in terms of log r, the same percentage of softening of apples and carrots will occur upon the application of similar increments of gamma radiation.

Both the average TD and the average CD_{50} values are significantly different for apples and carrots. Results reported here do not explain why variations are found in the TD and CD_{50} values of apples and carrots of the same variety or why apples and carrots show significantly different TD and CD_{50} values. Our original working hypothesis was that such variations may be accounted for on the basis of the degradation of pectin and cellulose in the tissue. We have now shown in model experiments that these two cell wall constituents are significantly degraded in the same dosage range as is required to soften plant tissues *(3, 4)*. However, further work is needed to clarify whether changes in the cellulose and pectin account for the differences in the behavior of different varieties of apples and carrots and for the significantly different behavior of apples and car-

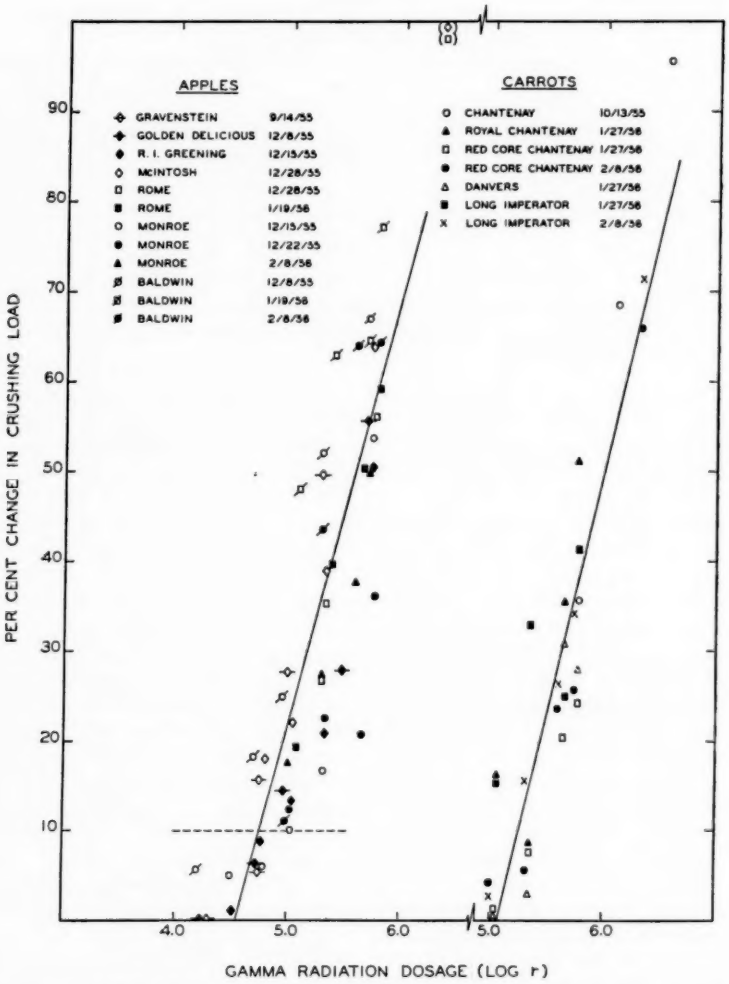

Figure 1. Relation between the natural logarithm of gamma radiation dosage and the percentage change in the crushing load for all samples of apples and carrots. The percentage change is expressed on the basis of the values for the unirradiated control tissue cylinders. The values below 10% change and indicating 100% change were not used in the calculation of the over-all line shown for apples. Equal value was given to the units of log r and percentage change in the computations but in order to accentuate differences, in Figure 1, the horizontal scale is 12.5 times that of the vertical scale. Therefore the angles in the drawing do not correspond to the calculated values.

rots. Differences in the radiation-susceptibility of these polyoses as formed in different tissues or variations in the protective action of other plant constituents present might possibly explain why one tissue has a different TD value than another. At this early stage of irradiation studies on plant tissues the possibility should not be excluded that physiological factors like changes in the turgor pressure in the tissue, for instance, might influence the irradiation-induced softening.

SUMMARY

The flesh of seven varieties of apples and five varieties of carrots was irradiated in the form of cylinders with gamma radiation from a Co^{60} source. The dosages were in the range 16.2×10^3r $- 2210 \times 10^3$r for apples, and 100×10^3r $- 2070 \times 10^3$r for carrots.

These dosages were sufficient to cause softening of the tissues. Firmness was measured by the load in pounds required to crush the cylinders. In the case of all tissues a linear relationship was obtained between the percentage change in crushing load and the logarithm of the gamma radiation dosage within these ranges.

Calculated threshold dosages (TD), defined as the minimum amount of radiation required to bring about a measurable softening, ranged from 4.2×10^3r to 107×10^3r for the apples, and from 23.5×10^3r to 178×10^3r for the carrots.

Calculated CD_{50} values, defined as the dosage required to cause 50% decrease in firmness, ranged from 123×10^3r to 834×10^3r for apples, and from 671×10^3r to 2310×10^3r for carrots.

Considering the over-all lines calculated from all results on apples and on carrots, the threshold dosages were 34.3×10^3r and 117.0×10^3r and the CD_{50} values were 400×10^3r and 982×10^3r, respectively.

The calculated angles that the lines representing the log r vs. percentage change in crushing loads form with the abscissa are almost constant. Over-all values are 88° 46′ for all apple irradiation tests and 88° 57′ for the carrot experiments. The near identity of the angles of the two over-all lines in Figure 1 indicates a possible common denominator in the irradiation-induced softening of apples and carrots.

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FREE RADICALS IN ROASTED COFFEE[a]

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Stable free radicals formed by the pyrolysis of carbonaceous materials at 250° C. to 600° C. (482° F. to 112° F.) have been detected in a wide variety of substances by their electron paramagnetic resonance (EPR) absorption *(1, 3, 4, 7, 8)*. It has been suggested that any organic material, if charred under the proper conditions, will show this absorption *(1)*. This note reports some preliminary EPR measurements which were made on several commercial vacuum-packed coffees to determine if free radicals are formed at the relatively low roasting temperatures employed commercially.

The free radicals in low-temperature carbons are thought to be associated with broken bonds in condensed carbon rings *(1)*. The concentration of these free radicals in many charred organic substances is strongly dependent on the charring temperature, rising to a maximum at about 550° C. (1022° F.) and then falling rapidly to zero above about 600° C. (1112° F.). The maximum free radical concentration at any given charring temperature depends on the length of time the material is heated, and also on the nature of the compound from which the char is formed.

The paramagnetic resonance absorption by the un-paired electrons in free radicals provides a very sensitive measure of free radical concentration *(6)*. This measurement can be made rapidly and nondestructively with a microwave electron paramagnetic resonance spectrometer. The free radicals can thus be detected in opaque solids without altering the test substance in any way. The sensitivity of the apparatus used in this investigation is approximately 10^{14} free radicals.

EXPERIMENTAL

A microwave EPR spectrometer of conventional design *(5)*, operating at a nominal frequency of 8800 megacycles per second, was used for these measurements. Ground samples and whole beans in thin wall capillary tubes or test tubes were placed in a transmission type microwave cavity resonator, and the rate of change of absorption of microwave energy by the sample was recorded while the magnetic field was swept slowly through the resonance value. The peak-to-peak distance between inflection points of the resulting absorption derivative curve was used as a measure of EPR absorption intensity, and, therefore, of free radical concentration. All measurements were made at room temperature.

Vacuum-packed, drip grind coffee, and roasted whole beans were purchased from retail sources for the EPR measurements. Green beans of the Santos variety were used in the roasting experiments. Roasting was carried out in a laboratory muffle furnace, with the beans being spread out on a screen to facilitate rapid heat transfer.

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RESULTS AND DISCUSSION

Samples of 8 different brands of vacuum-packed, drip grind coffee, one brand of instant coffee, one brand of demi-tasse coffee, and one brand of roasted whole beans were examined. An easily detectable EPR absorption was found in all of the above samples. The recorded EPR absorption derivative curves were similar to those found in coals and organic chars, consisting of a single line about 7 gauss wide. An EPR absorption curve for a typical roasted coffee is shown in Figure 1, in which the relative

Figure 1. EPR absorption derivative curve of roasted, vacuum-packed coffee.

EPR absorption (ordinate) is recorded with respect to increasing magnetic field (abscissa). Since, in the usual modulation method employing phase detection, the rate of change of absorption is measured, the recorded curve approximates the first derivative of the true absorption curve, and the cross-over point between the two inflection points corresponds to the exact center of the true absorption.

The EPR absorption intensities of the 8 coffees representing typical roasts were approximately equal. The free radical content was estimated to be in the order of 10^{16} per gram, based on a comparison of intensity measurements on known amounts of diphenyl-pieryl hydrazyl. The demi-tasse coffee, a very heavy roast, had a fourfold stronger absorption, while the instant coffee had a very much weaker absorption, than that of the typical roasts. There was no appreciable difference in absorption intensity

between roasted, ground coffee and the roasted whole bean. Green, unroasted coffee did not have a detectable EPR absorption.

The weak EPR absorption of instant coffee suggests that the free radicals are partially extracted by water. Coffee grounds remaining from brewed coffee were dried and examined for EPR absorption. A strong absorption, approximately equal to that of the original roasted coffee, was found, indicating that the free radicals are not appreciably soluble in hot water.

Further measurements were made on green coffee beans roasted for 15 minutes in air at temperatures from 200° F. to 500° F. The relative EPR absorption intensities are shown in Figure 2. Over the range of temperatures normally used in commercial coffee roasters (400° F. to 450° F.), the concentration of free radicals produced during roasting increases uniformly with increasing roasting temperature.

At any given temperature, the concentration of free radicals also depends on the length of time the sample is heated. The maximum free

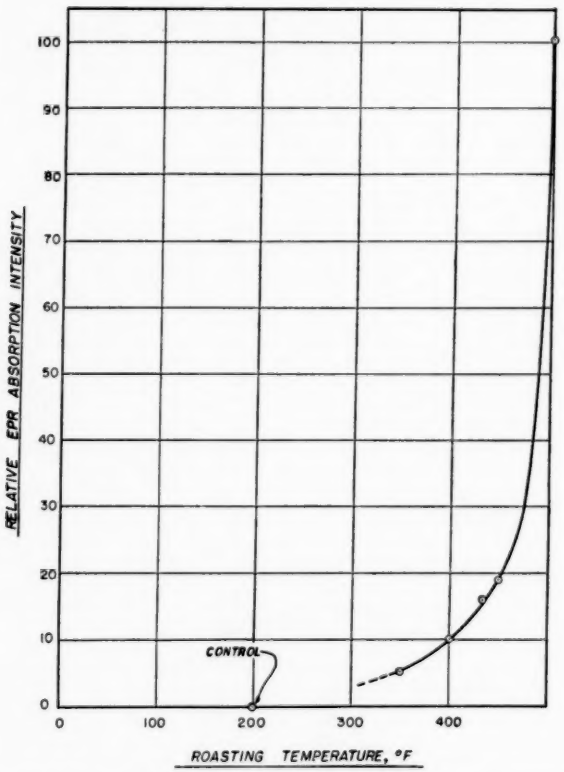

Figure 2. Variation of EPR absorption intensity with roasting temperature (15-minute roast).

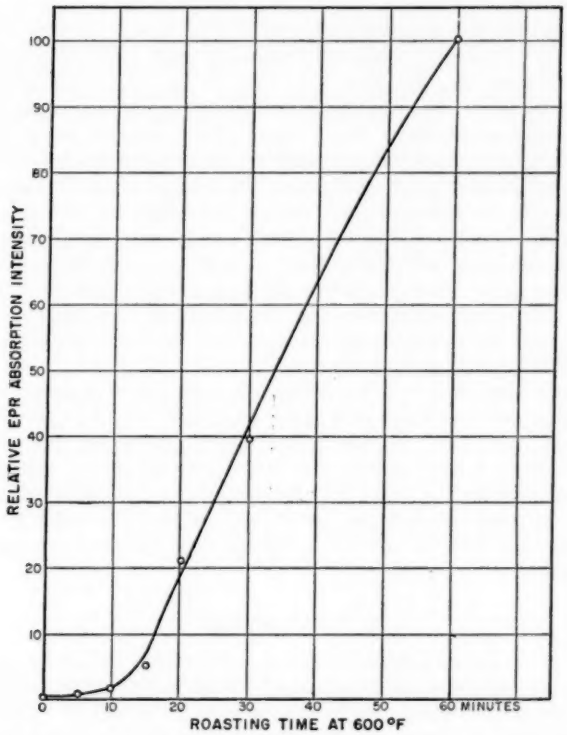

Figure 3. Variation of EPR absorption intensity with roasting time (600° F. roast).

radical concentration in charred dextrose is reached in a little over 2 hours *(7)*. The effect of roasting time on the free radical content of coffee is shown in Figure 3, in which are plotted the relative EPR absorption intensities for commercially roasted, whole bean coffee samples which were given an additional roasting in air at 600° F. for varying periods of time. The apparent induction period is probably only an artifact resulting from the length of time required for the whole beans to reach the roasting temperature.

The above roasting experiments do not, of course, duplicate the actual commercial roasting conditions, wherein the temperature of the coffee increases at a predetermined rate and the coffee remains at the final roasting temperature only momentarily before it is quenched. The data are presented only to illustrate the time-temperature dependence of free radical yield in coffee during heating. Under practical roasting conditions, the final free radical concentration would be expected to depend on the time-temperature history of the sample during the whole roasting process. A much more extensive study would be required to determine precisely how commercial process variables affect free radical yield and, more impor-

tantly, to determine whether free radical yield can be related to important quality factors in the roasted coffee.

The color of roasted coffee also darkens with increasing temperature and time of roasting, but the formation of the dark pigment does not appear always to be essential for free radical formation. Under some conditions of heating, we have found that a light colored roast will exhibit stronger paramagnetic resonance than will a somewhat darker roast.

No attempt was made to determine the minimum conditions of heating required for the formation of free radicals in coffee. However, a detectable paramagnetic absorption has been found in green beans heated at only 230° F. for one hour, indicating that at least some of the free radicals in roasted coffee arise from relatively low temperature reactions. Commoner *et al. (2)* have suggested that the formation of melanin-like condensed polymeric ring structures may account for the presence of stable free radicals in some biological substances.

We have also observed paramagnetic resonance absorption in other heated foods, such as toast and fried hamburg. It is probable that free radicals will be found in many food products which are heated to a sufficiently high temperature. Since the procedure for measuring free radical concentration is rapid and nondestructive, paramagnetic resonance absorption measurements might be useful for quality control during the roasting of coffee beans and in other similar thermal processes in the food industry.

CONCLUSIONS

The electron paramagnetic resonance absorption found in commercially roasted coffee indicates the presence of stable free radicals similar to those occurring in organic chars, coals, and in certain biological substances. The concentration of free radicals in coffee is dependent on both the temperature and length of the roast. The free radicals are present in very small amounts in instant coffee. They are also present, in approximately their original concentration, in coffee grounds after brewing, indicating that they are not appreciably soluble in hot water. The possible application of paramagnetic resonance absorption for quality control measurements is pointed out.

Acknowledgment

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SAFETY OF INSIDE ENAMEL COATINGS USED IN FOOD CANS

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Meetings between representatives of the Food and Drug Administration and metal container manufacturers pointed up these facts: (1) there was a lack of comprehensive evidence for the non-toxicity of inside can enamels; (2) incomplete knowledge of the chemical composition of the enamel film, and (3) wide use of inside enameled food cans by the American public. As a result, plans were made to conduct animal feeding studies with monkeys and rats on foods packed in cans with five representative inside enamel coatings[a] used in the largest volume for different classes of foods.

EXPERIMENTAL PROCEDURE

Canned foods fed. Selected canned foods were exposed to ten times the area of enamel with which they usually are in contact in commercially canned foods. This tenfold increase in the ratio of enameled area to product in the cans was obtained by coating extra strips of tin plate. The coated strips were corrugated, wound convolutely with flat sheets as separators, and inserted by hand into empty enameled cans. The corrugations with the interleaved flat sheets provided channels permitting complete exposure of enamel to the product (Figure 1).

All canned products fed to animals were packed in these specially manufactured cans. Products appropriate for the 5 enamels tested were selected for feeding: peas, red cherry juice, orange juice, beef, and liver. The peas, beef, and liver products were comminuted into a puree before packing. In discussing results, enamels will be identified by naming the product chosen for each enamel coating.

Direct feeding of enamel films which had been removed electrolytically from the can interior was objectionable for the following reasons:

(1) Some might question whether the enamel would remain in the intestinal tract long enough to result in extraction of soluble factors.

(2) Feeding large quantities of enamels might interfere with digestive processes.

(3) Ion exchange and absorption actions on vitamins, minerals, or other accessory growth factors known to be characteristic of related substances might take place in the intestinal tract.

Futhermore, the feeding of detached enamel film as a constituent of the ration sets up a condition that is entirely foreign to the normal use of canned foods. The feeding of canned foods which had been in contact with inside enamel coatings for suitable periods provides a more realistic evaluation of the films.

The canned products were stored for 3 months at 37.8° C. (100° F.) which is equivalent to one year's storage under normal warehouse conditions according to observations based on many years of experience with corrosion studies in plain and enamel lined containers. At the end of the 100° F. storage period the products were held at

[a] These enamels contained in their formulations the ingredients most widely used by the American Can Company in food cans.

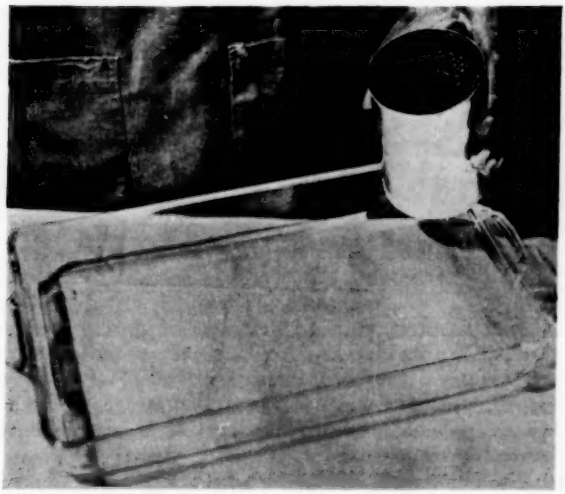

Figure 1. Experimental can containing corrugated inserts of coated plate.

−17.8° C. (0° F.) until fed. Control packs in unenameled cans were made for each product and stored in a similar manner. Animals were fed the canned food as a supplement to the regular stock diet at a level 10 times greater than that which occurs in the average American dietary, as calculated from statistics compiled by the United States Department of Agriculture *(5)*. Therefore, the resulting extractives from the inside coatings in the canned foods were fed to monkeys and rats at 100 times the level that would occur in the average American diet.

Care of monkeys. The Macaca mulatta colony was maintained in special conditioned quarters for 4 months prior to the inauguration of the experimental feeding program to accustom them to the stock diet and personnel. Tuberculin tests performed 2 months after arrival and every 6 months thereafter were negative. Animals were weighed weekly and handled daily so that any illness or injury could be promptly noticed and treated. Only occasional incidences of transient diarrhea were noted and no treatment was required.

The daily stock diet consisted of the following: ½ orange, 3 slices of whole wheat bread soaked in milk fortified with one ml. of cod liver oil per monkey, and kibbled dog food *ad lib.* One gallon of milk daily was used for the 36 monkeys. One g. of liver powder (1:20) dissolved in water was fed by stomach tube to each monkey weekly.

The animals were divided into 6 lots and received the following amounts of canned food daily: Lot I, orange juice, 32 ml.; Lot II, red cherry juice, 59 ml.; Lot III, pureed peas, 156 g.; Lot IV, pureed beef, 25 g.; Lot V, pureed liver, 25 g., and Lot VI, the controls, received no supplement. Animals were divided into the lots according to sex and weight so that there were no statistical differences in weights among the lots.

All canned food supplements were fed to the monkeys by stomach tube daily for 12 months. Complete blood studies including hemoglobin, red and white blood cell counts and differential blood cell counts were made every 3 months on blood obtained from the ear. A monkey holding box described in an earlier publication *(3)* enabled all these operations to be carried out with a minimum of hazard to both animals and personnel.

Care of rats. Rats of the Sprague Dawley strain were used. The animals were divided into 6 main lots with 5 sub-groups within each lot, and maintained in conditioned rooms. All rats in Lot I received 4 ml. of orange juice daily; those in Lot II, 7 ml. of cherry juice daily; those in Lot III, 23 g. of peas daily; those in Lot IV, 4 g. of beef

daily, and those in Lot V, 4 g. of liver daily. Lot VI was composed of controls which received no supplements. All the canned food was offered in auxiliary glass dishes which were emptied in approximately two hours.

Six rats in each lot were sacrificed after 3 months on test; six after 6 months on test; and twelve after one year on test, while 50 rats in each lot were assigned to the sub-group D which remained on test for 2 years. In addition, 20 rats were assigned to the sub-group E which received the control canned food supplement packed in the unenameled cans. There was no sub-group E in Lot VI. All rats received the stock ration and water *ad lib.* However, a record was kept of the stock ration consumed, namely:

Ground yellow corn	30%
Ground whole wheat	30%
Whole milk powder	30%
Linseed oil meal	3%
Alfalfa meal	3%
Dried liver powder	2%
Cod liver oil	1%
Bone meal	0.5%
Iodized salt	0.5%

The rats were weighed weekly and the same blood studies were made every 3 months as mentioned earlier for the monkeys.

Necropsy examinations. All animals were sacrificed at the end of the specified feeding period and macroscopic examination made of the viscera. The lungs, heart, liver, spleen, kidneys and gonads of all rats were weighed immediately upon removal from the animal. Tissue sections were taken on skin, striated muscle, thyroid, heart, lung, liver, spleen, pancreas, small and large intestine, kidneys, suprarenals, lymph nodes, urinary bladder, ovaries or testes, bone marrow and bone from both rats and monkeys.

RESULTS AND DISCUSSION

Monkey feeding study. All of the monkeys appeared in excellent health at the end of the experiment, most of them being sexually mature and having permanent teeth. The coats were thick and glossy. There was no evidence of alopecia, scaly dermatitis nor general unthriftiness. An analysis of variance indicated that there was a statistical difference in the body weights of the males and females at the end of the 12-month period, although there was no such difference in weights between animals receiving the canned food supplements and the controls. According to Schultz *(4)*, a sexual difference in body weight develops comparatively late in life and is in no way apparent during the first 4 years. It would appear, therefore, that these monkeys were over 4 years of age at the end of the test period. Examination of Table 1 indicates a lower value for the aver-

TABLE 1
Growth of monkeys fed supplements from enameled cans

Months	Lot I		Lot II		Lot III		Lot IV[1]		Lot V		Lot VI	
	Orange juice		Red cherry juice		Peas		Beef		Liver		Controls	
	Av.	Std. error	Av.	Std. error	Av.	Std. error	Av.	Std. error	Av.	Std. error	Av.	Std. error
0	2907 ± 163		2862 ± 115		2930 ± 126		2730 ± 115		2938 ± 124		2951 ± 95	
3	3510 ± 202		3336 ± 139		3486 ± 140		3257 ± 103		3570 ± 162		3437 ± 96	
6	3900 ± 233		3958 ± 197		3776 ± 108		3624 ± 118		4116 ± 216		3969 ± 221	
9	4298 ± 291		4308 ± 312		4239 ± 158		3850 ± 172		4876 ± 270		4601 ± 325	
12	4850 ± 392		4728 ± 383		4702 ± 227		4197 ± 165		5608 ± 316		5017 ± 365	

[1] Four females and two males in this group; all other groups have three females and three males.

age body weights of Lot IV than the other lots, but this may be explained in part by the distribution of four females and two males in this lot, whereas the other lots were evenly divided between the sexes. In addition, this lot was also the lightest at the beginning of the test.

A detailed discussion of the blood picture of the normal *Macaca mulatta* has been presented in an earlier publication *(3)*. At the 12-month examination, Lot II, receiving red cherry juice, as well as the control, Lot VI, had hemoglobin values significantly lower than the other four groups. The difference was 1.4 g. of hemoglobin per 100 ml. of blood. All the final hemoglobin values were lower than the initial values. An analysis of variance showed that there was no difference among the 36 monkeys in red blood cell counts due to sex or dietary supplement, but time or age of the animal did have a significant effect. A very significant decrease in the white blood cell count occurred after the initial examination. This decrease was explained on the basis of the "alarm reaction" *(3)*. These values are shown in Table 2. A lymphocytosis was found at the initial examination when the high white count occurred, but this condition disappeared when the white count decreased. Two individual monkeys had much higher eosinophil counts than the others, but no evidence of parasitic infection was found at necropsy. No explanation can be offered to explain the eosinophilia.

At the end of the 12-month feeding period all the animals were sacrificed and tissues taken for histological sections. Histopathological examination of the tissue from all monkeys revealed them to be normal. Some small cysts were seen at the medullary border of the adrenals, but according

TABLE 2

Blood pictures of monkeys fed supplements from enameled cans, with average and standard error

	Months	Hemoglobin gm/100 ml		Red blood cell count 10^6/mm^3		White blood cell count 10^3/mm^3	
		Av.	Std. error	Av.	Std. error	Av.	Std. error
Lot I							
Orange juice	0	12.5	± 0.32	4.38	± 0.088	14.9	± 2.27
	12	11.1	± 0.15	3.66	± 0.17	8.24	± 0.56
Lot II							
Red cherry juice	0	13.9	± 0.29	4.29	± 0.23	16.87	± 2.68
	12	10.1	± 0.35	3.96	± 0.23	7.89	± 0.50
Lot III							
Peas	0	13.5	± 0.50	4.46	± 0.18	18.42	± 1.74
	12	11.0	± 0.63	4.24	± 0.13	10.29	± 0.68
Lot IV							
Beef	0	13.8	± 0.61	4.36	± 0.095	12.71	± 1.57
	12	11.5	± 0.33	3.80	± 0.16	8.42	± 0.63
Lot V							
Liver	0	13.6	± 0.21	4.31	± 0.14	19.47	± 2.24
	12	11.2	± 0.38	3.93	± 0.14	8.42	± 0.50
Lot VI							
Controls	0	13.3	± 0.47	4.27	± 0.14	13.73	± 1.54
	12	10.2	± 0.35	3.94	± 0.18	8.82	± 0.48

TABLE 3

Weight gain, food consumption and efficiency ratio of rats fed supplements from enameled and plain cans for twenty-four months

	Lot I	Lot II	Lot III	Lot IV	Lot V	Lot VI
	Orange juice	Cherry juice	Peas	Beef	Liver	Controls
	Supplements from enameled cans (50 rats per lot)					
Total wt. gain/gm.						
Males	569	561	639	542	559	522
Females	447	396	412	348	349	329
Total calories						
Males	50,320	51,794	56,510	51,179	49,956	51,200
Females	30,048	43,342	46,078	41,687	40,916	40,936
"Efficiency" gms. gain/ calories × 100						
Males	1.13	1.08	1.11	1.06	1.12	1.02
Females	1.14	0.91	0.89	0.83	0.85	0.80
	Supplements from plain cans (20 rats per lot)					
Total wt. gain/gm.						
Males	530	504	535	487	595	.....
Females	338	402	324	316	320	.....
Total calories						
Males	51,108	52,546	55,694	51,103	52,196	.....
Females	40,200	44,386	44,550	41,675	39,588	.....
"Efficiency"						
Males	1.04	0.96	0.96	0.95	1.14	.....
Females	0.84	0.90	0.73	0.76	0.81	.....

to the literature *(2)* this is a normal condition in a monkey. Some foreign body granulomas were seen in the lungs.

Rat feeding study. For the sake of brevity, only the 24-month rat feeding data are presented in Table 3. The efficiency of the ration in terms of grams of weight gain per calorie consumed multiplied by 100, the total weight gain in grams, and the total calorie consumption of the various lots of rats were compared. If the data obtained on the rats fed supplements from the enameled cans are compared with those of the controls, Lot VI, it is apparent that the controls gained the least amount of weight and had the lowest efficiency rating. If intra-lot comparisons are made between the groups receiving the supplements from plain and enameled cans, the males and females receiving orange juice, peas, and beef and the males receiving cherry juice from the enameled cans gained more weight and had higher efficiency ratings. The males fed liver from enameled cans gained less weight but had the same efficiency ratings as those which received the supplements from the plain cans. The females receiving cherry juice and liver from both types of containers differed only slightly in their weight gain and efficiency rating.

There was little difference in hemoglobin values between the lots and subgroups within the lots, and the standard errors of the determination were relatively small. The red and white blood cell counts were similar to the hemoglobin values in that there was little difference among the values. These data are shown in Table 4. The initial lymphocyte values in the

TABLE 4

Hemoglobin, red and white blood cell counts on rats receiving supplements from enameled and plain cans for twenty-four months

	Lot I	Lot II	Lot III	Lot IV	Lot V	Lot VI
	Orange juice	Cherry juice	Peas	Beef	Liver	Controls
	Supplements from enameled cans (50 rats per group)					
Hemoglobin gm/100 ml						
Initial	13.4 ± 0.08	12.7 ± 0.10	11.4 ± 0.17	11.6 ± 0.28	12.0 ± 0.13	11.7 ± 0.43
Final	11.4 ± 0.20	11.0 ± 0.23	11.5 ± 0.18	11.9 ± 0.22	12.8 ± 0.22	13.0 ± 0.25
Red blood count 10^6/mm^3						
Initial	5.42 ± 0.10	5.16 ± 0.85	4.81 ± 0.09	4.33 ± 0.06	4.48 ± 0.05	4.20 ± 0.11
Final	5.57 ± 0.11	5.32 ± 0.13	5.39 ± 0.15	5.14 ± 0.16	5.05 ± 0.13	5.08 ± 0.17
White blood count 10^3/mm^3						
Initial	7.50 ± 0.13	8.88 ± 0.22	8.67 ± 0.16	8.29 ± 0.16	8.47 ± 0.15	8.28 ± 0.16
Final	8.04 ± 0.26	8.48 ± 0.26	9.02 ± 0.26	9.62 ± 0.44	9.29 ± 0.52	9.14 ± 0.48
	Supplements from plain cans (20 rats per group)					
Hemoglobin gm/100 ml						
Initial	11.1 ± 0.22	12.0 ± 0.46	10.0 ± 0.12	12.6 ± 0.21	12.6 ± 0.25	.....
Final	11.4 ± 0.28	10.7 ± 0.18	11.2 ± 0.15	11.2 ± 0.41	10.8 ± 0.20	.....
Red blood count 10^6/mm^3						
Initial	4.52 ± 0.12	5.23 ± 0.13	4.76 ± 0.14	5.36 ± 0.12	5.36 ± 0.59	.....
Final	5.28 ± 0.20	5.47 ± 0.19	4.85 ± 0.28	5.53 ± 0.15	5.54 ± 0.17	.....
White blood count 10^3/mm^3						
Initial	8.78 ± 0.36	8.60 ± 0.24	8.11 ± 0.28	8.70 ± 0.29	12.31 ± 0.34	.....
Final	8.61 ± 0.47	11.04 ± 0.19	9.18 ± 0.96	9.23 ± 0.59	10.38 ± 0.61	.....

differential counts were high for all groups of animals but fell gradually during the course of the experiment to the range of 60-70%. Since the combined percentages of the lymphocytes and polymorphonuclear neutrophils is equal to or greater than 90%, these latter values rose correspondingly during the course of the experiment. The percentages of monocytes and eosinophils were very low throughout the experiment. The monocytes range from 2.5 to 5.0% and the eosinophils from 0.1 to 1.0%.

The percentage survival rates for rats fed supplements from enameled cans ranged from 52 to 88% and for those fed supplements from plain cans the range was 30 to 90%. The survival rate for the controls was 68% for the males and 64% for the females. The incidence of tumors occurring in the rats on the long term test was extremely high. A total of 224 tumors was observed during the course of the experiment. Twenty-eight of these occurred in the males. The control group contained the greatest number of animals with tumors and the greatest number of tumors. The incidence for the controls was 1.7 tumors per female and 0.1 per male in the group of fifty. The highest incidence of tumors in an experimental lot was 1.5 tumors per female in the group fed orange juice from enameled cans, while the corresponding rate for the males from that lot was 0.02.

Histopathological examination of these tumor tissues demonstrated that they were fibro-adenomas. In addition, islet-cell adenomas of the pancreas and adenomatous hyperplasia of the adrenals and thyroids were found in three instances. Four instances of periarteritis and three instances of abscesses of the myocardium were also noted, but no malignancies were found. Cystic nephropathy was found in 25 males and 7 females from a total of 60 rats whose tissues were examined microscopically. Only 5 males and 23 females were free from this condition.

CONCLUSIONS

No differences in blood picture, weight gains, nor findings in gross and microscopic examination of the tissues were found in the 30 monkeys fed supplements from enameled cans when compared with the 6 animals fed the stock ration. These young growing animals were maintained for one year on force fed experimental canned foods without adverse effects even though the foods were fed at 100 times the level at which such enamel extractives could be expected to occur in the average American dietary. It would seem improbable that such extractives in the minute amounts at which they are known to occur *(1)* could present a public health hazard.

The 24-month rat studies lasted the average life span of the animal without effects traceable to the diet. The high incidence at which spontaneous tumors occurred in the control animals appeared to indicate a genetic weakness in the strain. Again there were no differences in food consumption, weight gain, or blood pictures among the 11 groups of rats and examinations of the tissues were negative. These facts add greater weight to the conclusion that inside enamel coatings for food cans as manufactured at the present time are not a public health hazard.

SUMMARY

A diet designed to evaluate the safety of inside enamel coatings for food cans was fed to rats for twenty-four months and to monkeys for twelve

months. No adverse effects caused by the diet on the body weights, blood picture or general health of the animals could be noted. Gross and microscopic examination of the tissues revealed no pathology. The evidence obtained in this study indicates that enamel coatings for food cans as presently manufactured are non-toxic.

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FLAVORS IN FOODS — A CUMULATIVE REVIEW

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Flavor in foods has always been recognized as a source of challenging problems. Recently, scientific and technological interest in flavor problems has been heightened. There was abundant evidence of this renewed concern at the flavor conference[b] held in Chicago in November under the joint sponsorship of the National Research Council and the Quartermaster Food and Container Institute. Reports of both subjective and objective studies were presented, and new vistas were opened on the elusive quality that gives to a food its characteristic appeal. Needless to say, the mysteries of flavor were not all revealed at this conference despite the many fine contributions. But those in attendance were impressed by the solid work currently being conducted, the new techniques being utilized to detect flavor mechanisms, and the many new approaches to understanding flavor —not the least of which are those relating to the physiological phenomena currently being explored. The question was asked at the end of the conference: what can be done to keep a cumulative check on flavor studies? In answer to this question the suggestion was made that a running review of the findings coming out of current flavor work should be maintained and published periodically in a scientific journal. The journal proposed was *Food Research*, wherein numerous articles on flavor have already appeared. Out of this discussion originated the present contribution. It is apparent to all of us who are interested in flavor that the task of accumulating the key findings in this ever-expanding field is difficult. The service can be perfected only by suggestions and contributions from the scores of scientists and technologists now absorbed in the study of flavor. Their assistance is herewith cordially invited.

The scope of contemporary food flavor research and technology is impressive. Starting with flavor as a psychological problem we can extend the list to problems in the physiology of flavor, the chemistry of flavor, and the technology of flavor. Each of these broad divisions can be broken down into numerous subdivisions since the manifestations of flavor can also be studied by the methods of histology, physics, bacteriology, and nutrition, as well as still other sciences. The time has not yet come perhaps when advances in flavor research can be integrated, but by bringing them all together the interrelationships will at least be more conveniently evident.

This department proposes at the outset to present in abstract form some of the work currently being done on flavor problems. To attain the

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[b] Proceedings of this conference are undergoing final editing and will be available at the Quartermaster Food and Container Institute for the Armed Forces, Chicago, within the next few months.

proper coverage, the cooperation of authors of articles, reveiws, or monographs will be required and, as mentioned, is herewith cordially invited. Particularly needed will be summaries of contributions to flavor research. It is suggested that these summaries be sent to the Quartermaster Food and Container Institute, marked to the attention of the compiler. Through this means as well as by searches of the literature conducted by this compiler and his associates[c] it should be possible to bring together in succinct form a wealth of information on flavor and to provide thereby a reference source and guide that will increase in usefulness with time.

This initial effort to compile the current findings of flavor research must be considered only as a point of departure. Its incompleteness is obvious. But a beginning has been made and unless all signs fail, the current lively interest in flavor problems assures the growth of this department. With growth, and with the anticipated cooperation and active interest of flavor specialists, it should become possible to furnish from time to time not only summaries but also critiques of progress in flavor studies. At this time, we shall only offer a recapitulation, in the form of brief abstracts, of a selection of recent appearances in the scientific and technological literature on flavor.

Chemical Aspects of Flavor by Commodities

Italian cheese ripening. IV. Various free amino and fatty acids in commercial Provolone cheese. W. P. Harper and J. E. Long. *J. Dairy Sci.*, **39**, 129 (1956). Free amino acid and free fatty acid content of commercial Provolone cheeses of the same age showed wide variations. Free fatty acid content varied in relation to the type of enzyme product used in its manufacture whereas the free amino acid content was apparently related to the type of starter organism. An interrelationship was noted between the concentration of free butyric acid and free glutamic acid and desired flavor intensity. About 1 mg. of free butyric acid and 2 mg. of free glutamic acid were found in cheeses in which characteristic flavor was noted. The concentration of each increased with flavor intensity, maintaining a ratio of approximately 1:2 of butyric to glutamic acid.

Ibid. V. Various free amino and fatty acids in commercial Romano cheese. *J. Dairy Sci.*, **39**, 138 (1956). Various free amino and fatty acids were measured in 14 samples of commercial Romano cheese. The amounts of some of the free amino acids could be related to the age of the cheese but only the free glutamic acid could be related to the flavor intensity of the cheese. Acetic and propionic acids were not associated with either the age of the cheese or flavor intensity, whereas both the higher acids and butyric acid were generally related to age and flavor intensity of the cheeses of the same age. In general, the free butyric acid and higher fatty acid contents were dependent upon the type of enzyme product used in the manufacture of the cheese, whereas the free amino acid concentrations were not associated with the lipase product added to the milk.

The coconut-like flavor defect of milk fat. I. Isolation of the flavor compound from butter oil and its identification as δ-decalactone. P. G. Keeney and Stuart Patton. *J. Dairy Sci.*, **39**, 1104 (1956). The coconut-like off-flavor compound, which develops in butteroil during storage or when butteroil has been heated, was isolated and identified as δ-decalactone (the lactone of 5-hydroxy decanoic acid). Infrared spectroscopy and paper chromatography were used in establishing the flavor compound's coidentity

[c] Dr. Stuart Patton, the Pennsylvania State University, and Dr. Lloyd Beidler, The Florida State University.

with δ-decalactone. Paper chromatographic methods are presented whereby a homologous series of γ lactones from nonalactone to dodecalactone can be resolved. Evidence that a similar series of δ-lactones can be separated in a like manner was obtained.

Ibid. II. Demonstration of δ-decalactone in dried cream, dry whole milk and evaporated milk. Coconut-like flavor extracts were obtained from dried cream, dry whole milk, and evaporated milk. The presence of δ-decalactone in these flavor extracts was established by paper chromatography, and the characteristic coconut-like flavor defect of these products is attributed to this lactone.

The neutral carbonyl compounds in blue-mold type cheese. M. E. Morgan and E. O. Anderson. *J. Dairy Sci.*, **39**, 253 (1956). A simple procedure for detecting the various methyl ketones in blue-mold type cheese is described. The method is based on paper chromatography of 2,4-dinitrophenylhydrazone derivatives prepared from cheese distillate. The method was applied to ten different samples of cheese, including French Roquefort, Danish and Domestic Blue, and Gorgonzola. The following carbonyl compounds were revealed in these cheeses: acetaldehyde, acetone, butanone-2, pentanone-2, heptanone-2, and undecanone-2. The three, five, seven and nine carbon methyl ketones were always present in cheeses possessing a typical aroma.

Radiation preservation of milk and milk products. II. Off-flavors in milk and cream induced by ionizing radiations as judged by organoleptic tests. G. W. Bierman, B. E. Proctor and S. A. Goldblith. *J. Dairy Sci.*, **39**, 379 (1956). Threshold doses at which off-flavor induced by high voltage cathode rays was recognizable in cream and various types of whole and skim milk range from approximately 7,000 to 25,000 rep, depending upon the product. The acceptability of the irradiated products decreased with increased dose. Homogenization, but not pasteurization by the high-temperature, short-time method, increased the resistance of whole milk to the development of off-flavor induced by ionizing radiations. Radiation induced off-flavors in milk and cream increased with increasing moisture and fat content. However, as the total solids increased, the product became less susceptible to flavor change.

A survey of various free amino and fatty acids in domestic Swiss cheese. P. H. Hintz, W. L. Slatter and W. J. Harper. *J. Dairy Sci.*, **39**, 235 (1956). Sixteen samples of Swiss cheese varying in age from 2 to 11 months were analyzed. Only cysteic acid, threonine-serine, glutamic acid and tyrosine-phenylalanine were present in all samples. Some free amino acids tended to increase in concentration during aging, while others remained relatively constant throughout the 11 months period. Butyric, propionic and higher acids were present in all samples studied, and valeric acid was present in all but one sample. There was no relationship between the age and the amounts of these acids present. An interrelationship between proline and propionic acid content and flavor was found. A minimum proline concentration of 2.0 mg. per gram and a minimum propionic acid concentration of 5.0 mg. per gram of cheese were present before the cheese possessed a satisfactory Swiss flavor. Values of either of these two acids below the minimum were associated with low flavor ratings.

Methyl sulfide and the flavor of milk. S. Patton, D. A. Forss and E. A. Day. *J. Dairy Sci.*, **39**, 1469 (1956). On the basis of gas chromatographic and mass spectrometric evidence, it is shown that the volatiles from milk contain methyl sulfide. Using 5 taste observers, the average flavor threshold value for this compound was observed to be 12 parts per billion in distilled water. The cowy-malty flavor and aroma of this compound is considered to contribute significantly to the flavor of fresh normal milk.

Frozen foods storage effects. Formation of alcohol, acetaldehyde, and acetoin in frozen broccoli tissue. P. A. Buck and M. A. Joslyn. *J. Agr. Food Chem.*, **4**, 548 (1956). The carboxylase activity of broccoli tissues was investigated to determine the role of pyruvic carboxylase in the production of volatile aldehyde and ketonic compounds which might serve as precursors of the off-flavors developing in underscalded frozen broccoli.

Broccoli carboxylase was found to catalyze synthesis of acetoin and diacetyl chiefly from added pyruvate and to a much smaller extent from acetaldehyde, unlike pea and wheat germ carboxylase. Acetaldehyde inhibited broccoli carboxylase activity. In frozen broccoli, both inhibition by and restricted diffusion of acetaldehyde would favor production of acetoin. The concentration of acetaldehyde, acetoin, or diacetyl was not related to the organoleptically objectionable formation of off-flavors, whereas ethyl alcohol content was related to extent of off-flavor.

Amines in cheddar cheese. G. J. Silverman and F. V. Kosikowski. *J. Dairy Sci.*, **39**, 1134 (1956). Cheddar cheeses were examined for free amines. Those definitely observed were cadaverine, putrescine, tyramine, histamine, tryptamine, and an unidentified amine present in relatively large amounts. The decarboxylation product of glutamic acid, gamma-amino butyric acid was also conspicuous in many of the cheeses. Levels of this compound generally increase with increases in the intensity of unclean flavor in the cheeses. Raw milk cheeses normally contained higher levels of amines than did pasteurized milk cheese. The presence of these amines is attributed to decarboxylase activity during ripening of the cheese.

Food irradiation. Sulfides released from gamma-irradiated meat as estimated by condensation with N,N-dimethyl-p-phenylenediamine. E. P. Marback and D. M. Doty. *J. Agr. Food Chem.*, **4**, 881 (1956). The reaction of hydrogen sulfide with N,N-dimethyl-p-phenylenediamine to form methylene blue has been adapted to determine small quantities of hydrogen sulfide from gamma irradiated meat. The odorous vapors from irradiated meat are carried with a stream of nitrogen into a trapping tube containing cadmium hydroxide and sodium hydroxide. The color is then developed by adding a mixture of the acid amine solution and Reissner's solution to a cup in the trapping tube, closing, and shaking. The intensity of the methylene blue color developed is then measured in a photoelectric colorimeter at 665 mμ. The method can be used for the quantitative estimation of 2 to 16 γ of hydrogen sulfide. Experiments show that free hydrogen sulfide probably is not present in gamma irradiated meat but that cadmium sulfide is formed in the trapping solution from some volatile sulfur-containing complex.

Flavors—natural and synthetic. Short, G. R. A. *Food Trade Rev.*, **26**(7), 3-6 (1956). Selection and use of both natural and synthetic flavors within the baking industry are discussed.

Broth-like flavor. Jacobs, M. B. *Am. Perfumer and Aromatics*, **67**(3), 59-60 (1956). Broth-like flavor is contributed by 3-methylmercaptopropionaldehyde. The author discusses the chemical and physical properties of this substance.

Flavor in instant coffee. Heyman, W. A. *Coffee & Tea Inds.*, **79**(9), 59 (1956). Development of soluble coffee is given in this article which is a part of a series. The first 10.5% of the weight loss during the roasting of coffee appears to be composed of objectionable green flavors. Last 4.5% of the loss contains flavor enhancers. Loss of volatiles that occurs during grinding amounts to 66%. These losses can be prevented in making soluble coffee, it is claimed, if the roast be quenched in cooled coffee concentrate such as is used to make the dry soluble powder.

Sensory Testing and Allied Subjects

Panel testing for flavors. Fourman, V. G. *Am. Perfumer & Aromatics*, **67**(5), 62 (1956). In selecting a panel to assure satisfactory results many factors must be considered. Techniques to be used in tasting pharmaceuticals for flavor and consumer panel testings are described.

A tasting experiment. Gridgeman, N. T. *Appl. Statistics*, **5**(2), 106 (1956). Two jam sugars were compared for preference and sweetness by a taste panel. Each judge tasted 7 pairs of jams (two flavors, four manufacturers). Analysis showed small but signifi-

cant differences in preference for and also in sweetness of the two sugars. Correlation between sweetness and preference was not obtainable. Some judges favored the sweeter and some the less sweet sample.

Lactone flavor components. Jacobs, M. B. *Am. Perfumer & Aromatics*, **68**(1), 53 (1956). The important lactones used as flavoring ingredients are discussed.

Effect of sucrose and organic acids on apparent flavor intensity. I. Aqueous solutions. Valdes, R. M., Hinreiner, E. H., and Simone, M. J. *Food Technol.*, 10(6), 282 (1956). A highly trained panel could distinguish between sweetness levels as the sucrose range increased but accuracy in detecting flavor differences decreased. A tendency to ascribe more flavor to the sweeter sample was noted. There was an optimum level (about 15%) beyond which excessive sweetness interfered with flavor judgments. There was a slight tendency to ascribe greater sweetness to the more flavorful solution. Samples that varied both in flavor and in sweetness gave inconclusive data.

Ibid., II. Fruit nectars. Valdes, R. M., Simone, M. J., and Hinereiner, E. H. *Food Technol.*, 10, 387 (1956). Nectars were evaluated by multiple comparison. The panel associated preference with the most flavorful sample, apparently; flavor intensity seemed to derive from the ratio of optimum soluble solids and optimum acidity; a high degree of accuracy was demonstrated in identifying the sweetest sample in all series. There was a dislike for the least sweet nectar in each series.

Physiology of Flavor and Related Topics

Carpenter, John. Species differences in taste preferences. *J. comp. physiol. Psychol.*, **49**, 139 (1956). Taste preference-avoidance behavior in rabbit, hamster and cat was studied. Quinine and KCl were avoided by all animals whereas preference for NaCl was shown by the cat and rabbit and avoidance by the hamster. The hamster and rabbit showed preference for sugar whereas the cat did not discriminate. Saccharin was also preferred by the hamster and rabbit and avoided by the cat. It was suggested that fluid intake is the result of interaction of taste and osmotic effect exerted by compounds ingested at isotonic levels.

Young, Paul Thomas, and Falk, John L. The acceptability of tap water and distilled water to nonthirsty rats. *J. comp. physiol. Psychol.*, **49**, 336 (1956). Nonthirsty rats selected tap water in preference to distilled water when given a choice. The same animals, when thirsty, showed no preference.

Adrian, E. D. The action of mammalian olfactory organ. *J. Laryn. Otol.*, 70, 1-14 (1956). An excellent review of the nature of the olfactory organ and its responses.

Kramer, C. Y. Additional tables for a method of choosing judges for a sensory experiment. *Food Research*, 21, 598 (1956). Tables for choosing a panel of judges for a sensory experiment to insure that the individual judges can detect differences at any given probability are reported.

Gridgeman, N. T. Group size in taste sorting trials. *Food Research*, 21, 534 (1956). Experimental trials indicate that small flavor differences are more efficiently detected when the coded samples are presented as 4 N pair comparisons than as N octads to be sorted by taste. The statistical theory of such trials is discussed. The tentative generalization is made that the larger the group presented to the subject in a taste trial the less efficiently can he exercise his ability to discriminate.

Liss, Leopold. The histology of the human olfactory bulb and the extracerebral part of the tract. *Ann. Otol., etc., St. Louis*, 65, 680 (1956). Silver-carbonate staining of the human olfactory bulb showed four distinct layers: 1) fila olfactoria; 2) glomeruli; 3) ganglion cells and 4) olfactory tract fibers. Several new types of structures have been described for the first time.

Engstrom, Hans, and Rytzner, Curt. The fine structure of taste buds and taste fibers. *Ann. Otol., etc., St. Louis,* **65,** 361-375 (1956). Each taste cell has 3-5 hair-like protrusions with a diameter of about 0.2 micron and length of 3-5 micra that enter the taste pore. The nerve fibers lose their myelin and then enter between but do not penetrate the cells of the taste buds. The entering nerves contain mitochondria-like formations as well as rich amounts of small granules or rods of 200-400 Å diameter.

Liljestrand, L., and Zotterman, Y. The alkaline taste. *Acta physiol. scand.,* **35,** 380 (1956). Electrophysiological studies show a response of the taste nerve of the cat to NaOH of pH 11.5 and above. The alkaline taste is a result of general stimulation of "water fibers," "salt fibers" and to some extent "quinine fibers."

Zotterman, Yngve. Species differences in the water taste. *Acta physiol. scand.,* **37,** 60 (1956). Electrophysiological studies show that the taste nerve fibers are activated by water in the cat, pig and rabbit but not the rat. The rat, however, is very sensitive to low concentrations of NaCl whereas the cat, pig and rabbit are not. Discrimination in the latter animals may depend upon a dual system of "salt" and "water" fibers which are reciprocally stimulated.

Guild, A. A. Olfactory acuity in normal and obese human subjects: Diurnal variations and the effect of D-amphetamine sulfate. *J. Lryn. Otol.,* **70,** 408 (1956). Olfactory acuity was greatest before a satisfying meal and least after. The increase in acuity before meals was eliminated if D-amphetamine sulfate was given 75 min. before meals.

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